

Malaria initiative needs drug industry's backing

The pharmaceutical industry must get behind recent efforts by science funding agencies to coordinate an international attack on malaria.

Celebrations to mark the fiftieth anniversary of India's independence from Britain (see below) have been dampened by the realization that the country is little closer today to the "greater triumphs and achievements that await us" than it was in 1947 when Jawaharlal Nehru, India's first prime minister, espoused these aspirations for the new nation in his independence address.

The irony of the parallel will not have been lost on the 650 scientists, public health officials and funding agencies meeting this week in Hyderabad, India, to celebrate the centenary of the discovery of the role of the mosquito in the malaria parasite life cycle. Hopes that the discovery would lead to comprehensive control of the disease have faded. One hundred years on, a vaccine remains elusive, drug treatment is stricken by resistance, and malaria still kills more than a million people every year.

This week brings a ray of hope for malaria research, however, with the decision by Britain's Wellcome Trust to invest £8 million in sequencing the genome of *Plasmodium falciparum*, the deadliest of the four malaria parasites affecting man. The sum is large in terms of the money available for malaria research worldwide, which at around \$100 million annually is some 25 times less than funding for AIDS. The grant also exceeds half the estimated costs of sequencing the *P. falciparum* genome, and combined with US funding, it assures the project's future.

Apart from having a broad impact on the whole field of malaria research, the availability of the parasite genome will have immediate payoffs in the discovery of new targets for drug discovery. Resistance

to the few available existing drugs is growing, and has already led to a tenfold increase in deaths from malaria in some parts of Africa (see *Nature* 386, 535; 1997). Moreover, the handful of new drugs currently being explored all come from just three families — quinolines, antifolates and artemisinin derivatives — all of which are prone to resistance. New targets and families are urgently needed.

But in the past few years, the pharmaceutical industry has all but abandoned drug discovery. Support for malaria remains strong in the research divisions of many companies, as well as among far-sighted managers who recognize that much of future sales growth will come from developing countries. But for now, it is the accountants who tend to have the upper hand.

The tightly focused and efficient international consortium that has coalesced around the malaria genome project should serve as a reminder to the industry that, with a little imagination and hard work, a relatively small amount of money can go a long way. The challenge now is for the pharmaceutical industry to find new means of overcoming the obstacles to restarting malaria research, by sharing the financial risks, for example. But industry leaders must first be willing to ringfence a modest amount of money for malaria research. The public relations payoff alone would probably balance the books.

Without adequate basic research, new leads for drugs and vaccines will quickly dry up. But increased political and financial support for this basic research will be forthcoming only if funding agencies can see product outlets for their research (see *Nature* 388, 211; 1997). The ball is now firmly in the pharmaceutical industry's court. □

Hope for science on the subcontinent

Fifty years after independence, India and Pakistan are still struggling to develop strength in science.

India and Pakistan are 50 years old, yet few in either country are in much of a mood to celebrate. Democratic India is racked with a degree of poverty that sits uncomfortably with its aspirations as an economic giant. Pakistan, despite higher economic growth, remains in the grip of political instability and near-feudalism.

The state of education, science and technology in both countries also leaves much to be desired. In Pakistan, half of the men and three-quarters of the women are unable to read or write. Fewer than 30 universities serve a population of 130 million, and produce fewer than two science PhDs per university per year.

So who is to blame? In a speech to the Pakistani scientific élite this month, the president, Farooq Ahmad Leghari, pointed the finger squarely at his audience, saying that scientists had let down the country by failing to deliver on their promises, particularly in agricultural research. Many of those assembled later greeted his remarks with laughter, and said that the president should be grateful for whatever science there is, given that the ministry of science's entire research budget is less than that of a medium-sized American university.

They have a point: since the 1960s, science policy has never been a

priority for any government of Pakistan. But, in other ways, the joke is on the scientists themselves. When the main advertised achievement of a country's principal research council is the development of a slim-diet, the government cannot be solely to blame.

India does better, spending around 1 per cent of its gross domestic product on science. On independence, it immediately set about setting up a research infrastructure, with 120 university institutes of technology and 100 national laboratories. Indian science has brought satellites, atomic power, increased food production, and has eliminated famine — something that had eluded its colonial masters.

For Pakistan, the road ahead will be a rocky one. Yet amid the gloom there are some grounds for optimism. The present, democratically elected government enjoys an unprecedented overall majority in the national assembly. The administration has a rare opportunity to break away from the past, and invest in education and research.

India's science base can only get stronger. It will continue to develop its strengths in biotechnology and in computer science, where it seeks to make the crucial transition from bulk software production to the supply of innovative products and services of its own. □



Funding assured for international malaria sequencing project

[PARIS] Britain's Wellcome Trust last week gave a much-needed boost to malaria research with a decision to invest £8 million in an international effort to sequence the genome of *Plasmodium falciparum*, the deadliest malaria parasite.

The three-year Wellcome Trust grant alone meets more than half the estimated \$20 million costs of the international project to sequence the 30 megabase genome of *P. falciparum*, and means that the project is now guaranteed to go ahead.

The grant is also substantial in terms of overall funding for malaria research, which amounts to only around \$100 million annually (see *Nature* **386**, 539; 1997).

The decision comes 100 years, almost to the day, after Ronald Ross, a British physician, discovered the role of the mosquito in the parasite lifecycle. To celebrate the anniversary, more than 650 researchers, public health officials and funding agencies are meeting this week in Hyderabad, India.

"The grant is a very big boost to the entire malaria field," says Dyann Wirth, a molecular parasitologist at Harvard School of Public Health who chairs the World Health Organization committee on malaria drugs. Malaria kills more than a million people every year.

The *P. falciparum* genome project is being coordinated by an international consortium, each body managing its own funds independently. Most of the sequencing work is to be carried out at the US Institute for Genome



The Sanger Centre: part of the consortium sequencing the genome of malaria parasite *P. falciparum*.

Research (TIGR), and the Sanger Centre at the Wellcome Trust's genome campus at Hinxton near Cambridge, England. Other participating institutes include the Naval Medical Research Institute (NMRI) in Rockville, Maryland, Stanford University, Harvard University, and Oxford's Institute of Molecular Medicine.

In addition to the £8 million grant from Wellcome, the US Department of Defense plans to invest \$8 million, while the Burroughs Wellcome fund has made a grant of \$2.5 million. The US National Institute of Allergy and Infectious Diseases (NIAID) has granted \$1 million to support pilot projects at TIGR and NMRI, and hopes to invest a further \$2 to \$3 million.

Each of the 14 chromosomes of *P. falciparum* has been assigned to an individual participant, and the genome is expected to be completely sequenced within three years. Initial funding has supported pilot projects aimed at developing techniques suited to the

P. falciparum genome, which is unusually high in adenine and thymine, two of the four nucleotides, or building blocks, that make up DNA. Sequencing of chromosomes 2 and 3, *P. falciparum*'s smallest chromosomes, have almost been completed in pilot projects at the TIGR and the Sanger Centre respectively.

The main immediate impact of the genome project is likely to be the discovery of new targets for drugs. Widespread resistance of the parasite to available drugs is emerging and research by the pharmaceutical industry to develop new families of drug has been all but abandoned (see *Nature* **386**, 540; 1997). Tim Stanley from Glaxo-Wellcome's malaria programme says the genome project "could open up 10 or 20 new families of drug".

The Wellcome Trust's decision marks a shift towards putting genome research at the heart of its infectious diseases programmes. To test the feasibility, the trust last year set up a Pathogen Genome Sequencing Centre at the Sanger Centre, choosing as pilot projects the bacterium *Mycobacterium tuberculosis* and chromosome 3 of *P. falciparum*.

The experience has convinced the trust that genome research is the most "cost-effective" means to support research in infectious diseases, says John Stephenson, the scientific programme manager, because it provides fundamental information for the global research community. "Genomics is going to change the whole way in which infectious disease research is done," says Stephenson.

Louis Miller, head of the Laboratory of Parasitic Diseases at NIAID, agrees. "For example, if we knew what molecule told the parasite it had hit a red cell, we could make a vaccine against it," he says. "By sequencing the parasite's entire genome, we will be able to identify the signalling genes involved in the recognition process."

But Adrian Hill, malaria vaccine researcher at the University of Oxford, argues that while genome research will provide new candidate antigens relevant to malaria in the medium term, the bottleneck in vaccine research remains the lack of funds for clinical trials of existing antigens.

Declan Butler

Europe's poorer regions woo researchers

[MUNICH] An initiative to encourage holders of 'trans-European' research fellowships to join industrial laboratories and laboratories in poorer regions of Europe is being backed by the European Commission (EC).

The Potential Host Institutes List (PHIL) has been set up so that laboratories can advertise their readiness to take young Marie Curie research fellows, who receive EC grants to work in European Union (EU) countries other than their own. The list is published on the World-Wide Web.

The initiative is an attempt to correct the uneven distribution of Marie Curie fellowships. The holders of one-third of the 2,200 fellowships awarded between 1992 and 1996 under the EC's Human Capital and Mobility Programme and its successor, the Training and Mobility of Researchers Programme, chose to work in Britain, a quarter in France, and 11 per cent in the Netherlands and Belgium.

In contrast, only 10 per cent went to Germany, and more peripheral EU countries were rarely chosen. Also, despite the EC's efforts to promote industrially relevant research, only two per cent of fellowship holders chose industrial host institutes.

The prime factor influencing the geographical distribution of host institutes appears to be the strength of research in central northern European countries. Germany's relative unpopularity is attributed to the perceived difficulty of the German language, as well as the continuing historical burden of the Nazi era.

Although the EC would like to see a more even spread, it is at present restricting support for PHIL to two of its political priority areas: industry and less-privileged EU countries. It will assess the effectiveness of the advertising of potential host institutes after the next deadline for applications in mid-September.

Alison Abbott

UK watchdog boosts science credentials

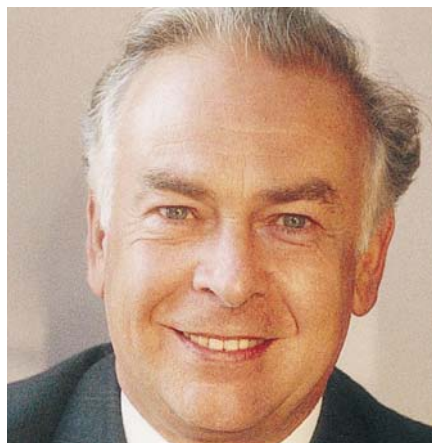
[LONDON] The gap between science and politics has narrowed slightly with the news that virtually all members of the new Select Committee on Science and Technology of Britain's House of Commons have qualifications in either science or engineering.

Indeed, some are arguing that the balance on the committee may have gone too far. In the previous Parliament, although most committee members had such qualifications, they were tempered by several non-scientists, primarily lawyers.

The five PhDs on the 11-member, all-party committee include the new chairman, Michael Clark (Conservative, Rayleigh), who took his doctorate at the University of Cambridge and started his career as a research scientist with the chemicals company ICI. He entered parliament in 1983.

Two of the other doctorates are held by Labour MPs who were members of the select committee in the previous parliament: Lynne Jones (Birmingham Selly Oak) in biochemistry and Alan Williams (Carmarthen East and Dinefwr), who holds a first-class honours degree in chemistry from the University of Oxford and later became a lecturer in environmental sciences at Trinity College in Carmarthen.

The remaining two PhDs are newly elected. Ian Gibson, formerly dean of biological science at the University of East Anglia, won the seat of Norwich North with an 11 per cent swing from the Conservatives, and Ashok Kumar, a chemical engineer specializing in fluid dynamics, who formerly worked for British Steel, won Middlesbrough South and



In the chair: Michael Clark, the new chairman of the House of Commons' science committee, is one of five of its members who have a doctorate.

Cleveland East for Labour, having fleetingly been MP for Langbaugh before the 1992 election.

Other new members of the select committee include Nigel Beard (Labour, Bexleyheath and Crayford), formerly a group research and development manager at ICI; Caroline Spelman (Conservative, Meriden), a food and biotechnology consultant; and Nigel Jones (Liberal Democrat, Cheltenham), formerly a computer programmer.

The expertise of the members means that they should, at least in principle, strengthen the committee's ability to fulfil its mandate to examine the "expenditure, policy and administration of the Office of Science and Technology and its associated public bodies".

Last month, the committee announced its first two inquiries. One will be into the review of further and higher education by the committee headed by Sir Ron Dearing (see *Nature* 388, 413; 1997); the second will examine potential problems faced by computers in the transition to the year 2000.

Some members of the committee are keen that it should play a key role not only in cross-examining government ministers and civil servants but also in pushing for new policy initiatives. "There may even be a need for a new white paper [policy document] on science," says Gibson, who stresses the need to raise the political profile of science in all major policy decisions.

The growing representation of scientists in parliament has generally been welcomed. "It seems to be an excellent thing," says Valerie Ellis, deputy general secretary of the Institution of Professional Managers and Specialists, the labour union that represents many government-employed scientists.

But others say that a scientific background does not necessarily provide the skills or knowledge to address hot political issues associated with research — for example, the patenting of genetic discoveries.

"It would be a pity if the views of the research community were to dominate the activities of the committee, which must be able to take lay perspectives into account," says one observer. "It's good that there is a strong presence on the committee of people who understand science and technology, but one might have preferred a slightly better balance."

David Dickson

Management problems prompt Canadian utility to shut reactors

[MONTREAL] Ontario Hydro, the largest power utility company in North America, is to close seven of its 19 Candu heavy-water nuclear reactors following an internal report that has heavily criticized the management of its nuclear division.

The company's president and chief executive officer, Allan Kupcis, has resigned, and its board has approved a major overhaul of its production facilities estimated to cost between C\$5 billion and C\$8 billion (US\$3.6 billion to US\$5.75 billion) over the next four years.

William Farlinger, Ontario Hydro's chairman, admitted last week that management practices appeared to have been deteriorating for the past 10 years. He said that the nuclear unit operated in the early years as "some sort of special nuclear cult". Farlinger said: "I don't think anybody understood the level to which our nuclear management had sunk."

Nuclear officials are keen to point out

that the report emphasizes that the problems lay with management, rather than with the technology. But Gary Kugler, vice-president for commercial operations at Atomic Energy of Canada (AECL), the company that designed and built the Candu reactors, said: "This is not welcome news."

AECL is planning to submit a proposal next month to sell two or more of its reactors to Turkey, and the company is trying to increase its sales to China, South Korea and Romania, which already have Candus. Indonesia, Vietnam, Thailand and the Philippines are also potential customers.

AECL officials are having to reassure such countries that the problems that led to the Canadian shutdown are not typical of the Candus.

Last year, nuclear power provided about 54 per cent of Ontario's electricity, down from 60 per cent or more several years earlier. In 1987, Ontario had six of the ten

top-ranked nuclear reactors in the world, but their problems have been growing in recent years. In 1983, a ruptured pressure tube at the Pickering plant cost about C\$1 billion to repair — more than the station's original cost.

Last December, the Atomic Energy Control Board, the nuclear regulator, produced a highly critical report on Ontario Hydro and gave the Pickering station an operating licence for only six months. In January, Kupcis brought in US nuclear experts to take over the utility's nuclear operations and report on its problems (see *Nature* 385, 287; 1997). It was their report that led to his resignation.

It is not clear whether all the reactors that are being shut down will eventually reopen. Those that do reopen will require upgrades, and any reactors that are closed permanently will have to be replaced by more expensive fossil-fuel plants.

David Spurgeon

\$100m payout after drug data withheld

[WASHINGTON] A pharmaceutical company has agreed to pay nearly \$100 million to settle potential legal claims from an estimated five million patients who have taken the anti-hypothyroidism drug Synthroid.

Knoll Pharmaceutical of Mount Olive, New Jersey, a subsidiary of the giant German group BASF, was charged with costing the patients more than \$2 billion because it suppressed research findings that showed Synthroid was no more effective than cheaper competitors.

Knoll announced earlier this month that it would pay at least \$98 million to plaintiffs rather than fight the charges in court. This appears to be the first time that patients have won compensation based on a sponsor's treatment of the results of a research trial.

More than a dozen class-action lawsuits have been filed against Knoll, alleging violation of state consumer protection and fraud statutes, as well as federal anti-trust and racketeering laws.

Knoll is accused of suppressing publication of a study that showed that one brand-name and two generic competitors were as effective as the more costly Synthroid. The study concluded that health-care costs could be cut by \$356 million a year if cheaper equivalents were used instead of Synthroid.

The British company Boots, which owned Synthroid before Boots' drug division was taken over by BASF in 1995, had threatened to sue Betty Dong, a clinical pharmacist at the University of California, San Francisco (UCSF), who led the study, because she had signed an agreement not to publish results without its consent.

The threat came just before an article by Dong went to press in the *Journal of the American Medical Association (JAMA)* in 1995, and Dong withdrew her paper after officials at UCSF refused to back her and her colleagues.

Before lawyers' fees are deducted, this month's settlement would provide about \$19.60 to each of five million people eligible for damages. The company says it will pay up to \$135 million if more than five million plaintiffs come forward.

Knoll insists that the settlement "in no way implies or acknowledges any wrongdoing". Knoll's president Carter Eckert says it was agreed "to avoid burdensome and expensive litigation".

Consumer activists complain that the settlement is too small. Paying each patient less than \$20 is "absolutely an outrage", says Sidney Wolfe of the Public Citizen Health Research Group.

Drummond Rennie, a deputy editor of *JAMA* and a professor of medicine at UCSF, agrees. He calculates that Knoll will pay out less than 5 per cent of an estimated profit of

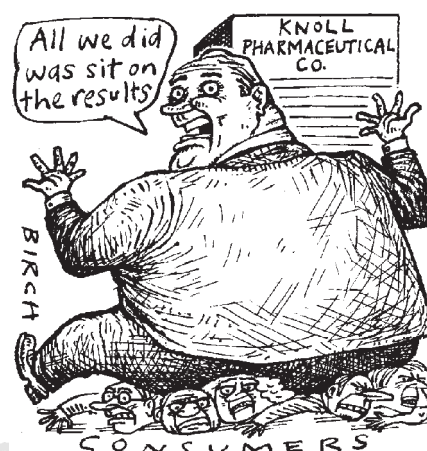
\$2.1 billion that it made by suppressing the findings between 1992 and 1997. "I'd feel pretty cheery if I were the company," wrote Rennie in an editorial accompanying the Dong paper's eventual publication in April.

But Barry Himmelstein, a lawyer for the plaintiffs at the San Francisco firm of Lieff, Cabraser, Heimann, and Bernstein calls the settlement "unusually good", and says he expects plaintiffs to recover "a substantial portion of their potential losses".

Some bioethicists say that the agreement sets an important precedent. Arthur Caplan, director of the Center for Bioethics at the University of Pennsylvania, says it is the first time in the history of US science that a group of patients has filed a class-action suit alleging injury because of the way in which research findings have been handled. "It shows that patient groups can get redress when they feel they have been mistreated in the context of a research trial."

The case does not, however, set a precedent in a strictly legal sense, as it was resolved out of court. And experts point out that, had it gone to trial, Knoll would have had a legal defence because of Dong's written agreement with the company.

Some observers feel that the company settled to avoid protracted bad publicity. But they say that the settlement is still likely to send a warning to the pharmaceutical indus-



try. Wendy Mariner, professor of health law at Boston University, says that companies could choose to avoid vulnerability to similar lawsuits "by saying that anyone can publish anything he or she wants".

Bioethicists and lawyers also stress that the episode should warn researchers against signing agreements with industry that cede rights to publish. They say that the behaviour of officials at UCSF in refusing to put legal resources behind Dong should be an object lesson. "It makes the researchers look expendable. That's a danger if you wish to have credible research emerge from universities," says Mariner.

Meredith Wadman

'Action needed to counter bioterrorism'

[WASHINGTON] The United States is failing to employ the power of its biotechnology and pharmaceutical industries to counter a growing threat from biological terrorism, an expert conference was told last week.

Biological terrorism is becoming more likely as both information and biological agents become more available, said David Siegrist, a research fellow at the Potomac Institute for Policy Studies, which hosted the conference in Washington, DC.

"There is a crying need for much more to be done," said Siegrist, who leads a research team studying how to counter biological terrorism. "The US advantage in biotechnology needs to be leveraged, so that it outpaces the threats."

The United States is not as well prepared to counter the threat as it should be, according to Siegrist's research. A 1996 report from the Federal Bureau of Investigation concludes that, although terrorist incidents in the United States are decreasing in number, they are increasing in destructive power and casualties inflicted. "Over the past ten years a pattern of interest in biological agents by criminals and extremists has developed," says the report.

Siegrist's team argues that increased funding for biomedical research into advanced countermeasures is needed. The lack of incentives for industry to invest means that government must do more.

A recent official review of US defence policy concluded that protecting Americans from weapons of mass destruction is a top priority, yet recommended that just \$1 billion over five years be put into developing tools to counter the impact of domestic attacks by such weapons.

But Stephen Morse, the manager of a new advanced diagnostics programme for pathogens at the US Defense Advanced Research Projects Agency, points out that the agency has boosted its funding for countering biological threats to \$50-\$60 million in the past few years. This is a "significant commitment", he says.

Technologies being developed by the government include biosensors — tiny devices that can identify biological agents in the body or in the environment. Advocates of increased research point out that there are many potential applications in the civilian sector, from food safety inspections to countering infectious diseases.

M.W.

Ecologists urged to 'win climate debate'

[ALBUQUERQUE, NEW MEXICO] Bruce Babbitt, the US Interior Secretary, last week urged 3,000 scientists gathered for the annual meeting of the Ecological Society of America to fulfil their "civic obligation" by helping to convince the American public of the case for man-made global warming.

"We have a scientific consensus but we don't have a public consensus" that man-made global warming is taking place, Babbitt said. "We can't get the message through by speeches from people like me. It is all of you that have that obligation."

Babbitt acknowledged scientists' tradition of caution when entering political debates. "But there are times that cry out for scientists' involvement, because the public doesn't have anywhere else to turn to."

During the past month, the administration of President Bill Clinton has embarked on an effort to win over an uninterested American public and a sceptical Congress to the idea that the United States should agree to binding limits on greenhouse gas emissions at December's meeting of nations in Kyoto, Japan (see *Nature* 388, 407; 1997).

Despite the fact that the administration has still failed to specify targets for emissions, as it was required to do in June under the international agreement known as the Berlin mandate (see *Nature* 374, 584; 1995), Babbitt called for ecologists' support in this belated charm offensive.



Babbitt: 'civic obligation' on scientists to convince the US public over man-made global warming.

But Babbitt pleaded for ecologists' forbearance on two other issues — forest management and the Endangered Species Act — on which the administration has taken compromise positions that anger some conservationists. He said that some forests need thinning to prevent fire. And he asked the audience to support the process under way in the Senate to reauthorize the act in a way that may insure property developers against future liability (see *Nature* 388, 506; 1997).

Babbitt declined to comment afterwards on reports that the United States and Japan are pushing Europe to withdraw its ambitious proposal for a reduction of greenhouse gas emissions of 15 per cent from 1990 levels by the year 2010. He also denied that the

administration was moving too late to shift public opinion. "The important thing is what happens from here forward," he said.

Jerry Melillo, associate director for the environment at the Office of Science and Technology Policy (OSTP) at the White House, backed Babbitt's plea. He called on ecologists to rebut a recent guest editorial in the *Washington Post* by Robert Eaton, the chairman of Chrysler, which played down the importance of car exhausts in climate change by arguing that 97 per cent of carbon dioxide in the atmosphere comes from plants, and less than 0.5 per cent from vehicle exhausts. While true, the statement is not relevant to the question of what has caused the recent sharp increase in carbon dioxide levels in the atmosphere.

Melillo said: "It is our responsibility as scientists to make sure that this kind of problem is straightened out."

OSTP is arranging workshops around the United States at which scientists, politicians and others will assess the impact of climate change and possible responses to it.

But Steven Sanderson, a political scientist and vice-president of Emory University in Georgia, expressed doubts about Babbitt's call for help. "There's an invitation here to turn science into politics, and I think there's a problem with that. The contribution scientists have to make is to do good science, not to become policy wonks." **Colin Macilwain**

Miami AIDS researcher denies charges over billing irregularities

[SAN DIEGO] A prominent AIDS researcher in Florida is facing trial in federal court in Miami over a billing scheme that started in 1989 in which he is alleged to have obtained illegally more than \$570,000 from two institutions.

Lionel Resnick, 42, who formerly ran the laboratory in the Pearlman Research Center of Mount Sinai Hospital in Miami Beach, was indicted last August in the US District Court in Miami on 52 counts of mail fraud and money laundering. He is alleged to have set up a mock laboratory company operating from his home to bill the University of Miami and the All Children's Hospital in St Petersburg, Florida, for tests related to AIDS research and clinical care.

In 1994, Resnick took a high-profile and controversial stand on whether a Florida dentist with AIDS had infected some of his patients with HIV. On the CBS programme *60 Minutes*, he challenged the view of the US Centers for Disease Control and Prevention that the dentist had infected the patients.

Resnick has pleaded not guilty to the charges against him and has placed about \$500,000 in an escrow account to pay back

the two institutions. His attorneys have described the federal indictment as an excessive action, and say that it grew from an administrative misunderstanding. The beginning of the criminal trial has been repeatedly delayed as Resnick contested legal points. An appeal to a higher court has delayed the trial until later this year.

The discovery of billing irregularities caused considerable concern at the University of Miami, where Resnick was a key collaborator on national AIDS trials, and led to a review of research projects in which he was involved. A dermatologist by training, Resnick carried out tests for AIDS research by Margaret A. Fischl, an internal medicine specialist at the university.

University officials say they found no problems with the scientific findings. The National Institutes of Health's Office of Research Integrity is monitoring the Resnick affair; officials there declined to comment. Resnick has left Mount Sinai, but continues to treat patients with AIDS-related lesions.

According to the federal indictment, the Mount Sinai laboratory headed by Resnick was set up with a \$1-million grant from the

state of Florida to carry out sophisticated tests for AIDS research. In July 1989, Resnick founded Vironc Inc., a private company with neither employees nor equipment. By October 1989, says the indictment, he was submitting invoices "to the University of Miami for payment to Vironc rather than Mount Sinai".

It continues: "In truth and in fact, the AIDS-related testing requested by the University of Miami continued to be performed at the Mount Sinai lab by Mount Sinai personnel using Mount Sinai equipment." By February 1990, Resnick is alleged to have done the same thing for tests from the All Children's Hospital.

Federal investigators have also been looking into billing practices at a health spa that Resnick owns in Miami Beach. Court documents say agents raided the Imagen Medical Day Spa in May 1996, seizing records after allegations that the federal Medicare health insurance programme for the elderly may have been improperly billed \$1.1 million for cosmetic treatments. No charges have been filed in this case, say federal officials. **Rex Dalton**

US—Cuba row over insects goes to weapons meeting

[PARIS] The countries that have signed the 1975 Biological Weapons Convention will meet next week in Geneva to hear Cuban allegations that last year the United States discharged the insect pest *Thrips palmi* over Cuba to damage the country's agriculture. The US State Department dismisses the allegations as "outrageous".

The meeting, requested by Russia on behalf of Cuba, will be the first time that the parties to the convention have heard allegations of an infraction of the convention.

But the meeting has no powers to make further investigations or impose sanctions, because the convention lacks a legally binding verification regime, unlike the Nuclear Non-Proliferation Treaty and the Chemical Weapons Convention. Efforts to add such a regime to the treaty have only recently started (see *Nature* 388, 317; 1997).

At most, the meeting could recommend that the United Nations Security Council request an investigation of the allegations, although neither the United States nor Cuba would have any obligation to comply. One official from the US State Department says, however, that the United States would comply. It is "confident of its case", and wants to act in the spirit of the treaty.

The controversy provides a clear example of why a verification regime is badly needed, says Alistair Hay, a chemical pathologist at the University of Leeds in the United Kingdom, and an expert on chemical and biological weapons. Such a regime could have allowed an international inspection team to be sent to Cuba at short notice.

Use of biological weapons is "very difficult" to prove at the best of times, says Hay, because their effects may be indistinguishable from natural outbreaks of disease. Adding to the uncertainty is the difficulty of verifying claims so long after the alleged incident, which is said to have occurred on 21 October 1996.

Cuba alleges that on this day a State Department aircraft designed to eradicate narcotics crops, which had been authorized to fly across Cuban airspace to Colombia, sprayed a substance over an area known as

the Girón corridor, where a *Thrips palmi* epidemic broke out a few weeks later. The aircraft had been seen releasing an unidentified substance by the pilot of a civilian Cuban airliner flying 300 metres below it (see map).

The State Department says that the pilot had released only smoke to signal its presence to the Cuban aircraft, and was following "safe and prudent" aviation procedures. Cuba argues that the Cuban pilot knew the difference between smoke and a substance. It contests that the US pilot needed to signal its presence, arguing that the flight plan gave responsibility for aircraft separation to air traffic control. Cuba questions the US assertion that all its narcotics control aircraft are fitted with smoke generators.

Hay points out that Cuba's case relies heavily on circumstantial evidence. Its position may be further weakened, he says, by the fact that *Thrips palmi* has spread throughout the Caribbean since it first appeared there in 1985, and is now found in Jamaica, the Dominican Republic and Haiti.

But Dorothy B. Preslar, of the Federation of American Scientists' programme on chemical and biological weapons, says that the insect would have been expected to have appeared on the east of the island and not in the west as in fact occurred, given the geography and prevailing weather. But she says that this could be accounted for by scenarios other than deliberate infestation.

Many observers are sceptical that the United States would take the political risk of using biological weapons, because it has been a leading proponent of efforts to reinforce the Biological Weapons Convention with a verification regime.

Yonah Alexander, director of the terrorism studies programme at George Washington University in Washington, DC, dismisses the Cuban accusation as "propaganda" intended to put the United States in a "no-win situation". Whatever the United States says, doubt will be cast on its credibility, Alexander says.

Others argue, however, that the United States's record of covert operations in Cuba leaves room for speculation. **Declan Butler**

Royal observatory could return to Greenwich site

[LONDON] Britain's Royal Greenwich Observatory (RGO) may — at least in spirit — be returning to the Thames-side site on which it was established more than 300 years ago, following the government's decision to close its current site in Cambridge and merge its research-related activities with those of the Royal Observatory in Edinburgh (see *Nature* 388, 105–106; 1997).

The observatory's present managers are drawing up a business plan under which the RGO's expertise in telescope design and technical support would be retained in a single non-profit organization. This would remain at Cambridge, and offer a telescope design consultancy service with Liverpool John Moores University, which provides telescope-manufacturing facilities.

The two institutions are already involved in designing and manufacturing a 2-metre telescope for India, and are keen to extend their collaboration on a commercial scale.

At the same time, the National Maritime Museum, which runs the museum on the site of the original observatory in Greenwich in southeast London, is keen to use the name to boost its highly regarded public education programme in the history of astronomy. The old observatory receives 400,000 visitors a year, and many astronomy enquiries from members of the public, who still think that Greenwich houses an active observatory.

Paul Murdin, head of astronomy at the Particle Physics and Astronomy Research Council (PPARC) and a member of the board of trustees of the maritime museum, says the two proposals could be linked under a single umbrella, with the RGO's scientific and technical expertise remaining in Cambridge, and the expanded public education activities run from Greenwich.

Jasper Wall, the RGO's director, says he is not against such an idea, but would want to ensure that a prominent link with the maritime museum does not overshadow the RGO's research. "The RGO is not a museum, and has never been one," he says.

One project being considered by the maritime museum is the building of a 2-metre telescope in Hawaii. This could be used via satellite by computer terminals in schools in the United Kingdom, or in the Greenwich museum. The 12-hour time difference would mean that the Hawaiian night sky could be seen in daylight hours in Britain.

PPARC is at present responsible for managing both royal observatories. Ken Pounds, professor of astronomy at the University of Leicester and PPARC's chief executive, says that a decision will be made next year. **Ehsan Masood**



Science comes back into fashion in Britain's schools

[LONDON] The number of UK school pupils taking science subjects at Advanced level (A-level) unexpectedly increased this year amid a decline in popularity for other 'traditional' academic subjects, the results of the 1997 examinations published last week show.

UK pupils study an average of three subjects at A-level for two years between the ages of 16 and 18. This year, more pupils sat for examinations in biology, chemistry, physics and mathematics than in 1996. Biology entries increased the most — by almost 9 per cent — from 51,894 last year to 56,534 in 1997. Chemistry entries increased by almost 5 per cent to 42,458, and physics by 2.2 per cent to 33,508.

This contrasts with last year when the numbers of candidates sitting A-level biology dropped by 16 per cent from the 1995 figures. The numbers for physics and chemistry also declined in 1996, by 5.65 per cent and 4.3 per cent respectively.

Robert Fairbrother, professor of science education at King's College London, says the popularity of biology A-level is also being reflected in this year's intake for science teacher training courses at King's College. Out of 64 students enrolled, 40 are biologists,

16 are physicists and the remaining eight chemists. Overall, this year, A-level entries rose 5.2 per cent to 776,115. A record 34.9 per cent of candidates achieved the highest grades of A or B.

Hubble spectrograph gets Boulder design

[LONDON] The task of designing a new \$25-million spectrograph for the orbiting Hubble Space Telescope has gone to a team led by astronomers from the University of Colorado at Boulder astronomers. It will be jointly built by the university and Ball Aerospace & Technologies Corporation in Boulder.

The Cosmic Origins Spectrograph will gather ultraviolet light from distant stars, galaxies and quasars in an attempt to better understand the origins of large-scale structures in the universe, and the physical conditions present early in its history. The instrument is planned to be installed during the final Hubble servicing mission in 2002.

BSE rules offend US renderers . . .

[BRUSSELS] After beef hormones and genetically modified foods, the United States and Europe look set to clash again over what constitutes scientifically sound precautions to protect public health. The US rendering

industry intends to sue the European Commission (EC) over regulations brought in to protect European consumers against bovine spongiform encephalopathy (BSE).

US renderers allege that an EC ban on the cattle parts judged most at risk has no scientific basis, and discriminates against US exports of tallow worth \$108 million a year. The United States, which has threatened to take the case to the World Trade Organization, says that unless the rules are changed, it would be obliged to bring its abattoir regulations into line with those in the European Union.

. . . while cattle semen comes under scrutiny

[LONDON] Members of a European Commission scientific advisory committee have expressed doubts about the safety of cattle semen, and have called for a review of the only UK beef product exempt from the European Union's worldwide ban.

The committee has asked the Berlin Institute for the Protection of Consumer Health to investigate whether bovine spongiform encephalopathy (BSE) can be transmitted through bovine semen and embryos. The institute's findings will be presented to a meeting on 8 September.

Doubts about embryos were initially raised by UK research, which showed that

BSE can in a minority of cases be passed from cow to calf. The mechanism of this transmission remains unknown.

ITER design on course, say its backers

[MUNICH] The design of the International Thermonuclear Experimental Reactor (ITER) is on the right track, ITER's council said in approving the interim report of its design teams in Finland last month. The council, which includes representatives from the governments of ITER's four partners — the United States, the European Union, Japan and Russia — also said it supported extending the Engineering Design Assessment (EDA) phase for three years to 2001, rather than moving into the construction phase when the EDA phase runs out next July. A delay to the construction phase would give the partners time to decide on a site for ITER, and, more importantly, to ride out a financial climate that remains hostile to the cost of the project, whose construction is estimated at around \$10 billion.

Lewis satellite focuses on remote sensing

[WASHINGTON] A satellite carrying advanced Earth-imaging instruments and subsystems intended to advance the state of the art in

scientific and commercial remote sensing was due to be launched earlier this week from the Vandenberg Air Force Base, California.

The Lewis satellite, built by TRW Space & Electronics Group under the US National Aeronautics and Space Administration (NASA)'s Small Spacecraft Technology Initiative, is one of several focused small missions developed under NASA's Mission to Planet Earth. It features remote-sensing instruments designed to analyse the spectrum of light energy reflected by Earth's land surfaces into up to 384 distinct bands, and also carries the Ultraviolet Cosmic Background astrophysics instrument built by the University of California at Berkeley.

Four new networks launched by ESF

[LONDON] The European Science Foundation has launched four more scientific networks to foster collaboration between European scientists and research institutes involved in the physical, life and social sciences, and the humanities. These four networks add to the existing 14, and will run for three years.

The topological defects network will cover non-equilibrium field theory in particle physics, condensed matter and cosmology. The alpine biodiversity network will study the flora and fauna of European mountains. The European theatre iconography network

will aim to correct literary bias in European theatre history. And the human reasoning and decision-making network will bring together social and cognitive scientists to improve understanding of this field.

German research faces crisis in chemistry

[MUNICH] New figures on student numbers published by the Society of German Chemists (GDC) indicate that German universities and research institutes as well as industry will soon face problems finding graduates to fill vacant research positions.

The current situation of too many young scientists for too few jobs could be reversed within six to eight years, concludes the GDC, as fewer students choose to study chemistry at university. Numbers have fallen by a third to around 26,000 in the past five years, and the number of PhD students, now more than 2,000, is predicted to more than halve.

Arnulf-Dieter Schlüter, head of the Institute for Organic Chemistry at the Free University in Berlin, is pessimistic. "With fewer graduate students, we will publish fewer papers which will result in less research funding," he says. "It is a vicious circle that could cause university research in chemistry to dry up." Industry should encourage the study of chemistry by making clear that employment prospects will be good, he says.

Public-sector patents on human DNA

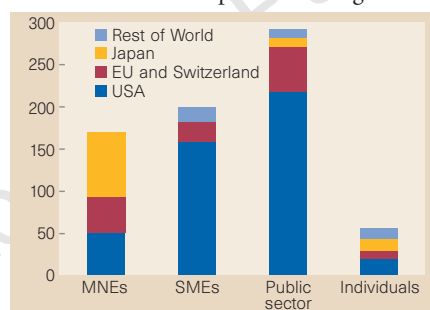
Sir—The increasing emphasis of genomics in therapeutic and diagnostic research and development (R&D) suggests a continuing dominant role for the pharmaceutical industry in patenting human DNA. Here we present new data that challenge this broadly accepted trend by revealing that patenting activity of human DNA sequences by the US public sector is now approaching that of the private sector worldwide.

We have used patents published after filing in order to reflect more recent R&D activity. Our methodology used the GENESEQ database (Derwent Ltd, UK) to analyse patents published in 1995 that included claims for human DNA sequences. (About two-thirds were Patent Convention Treaty applications, the remainder being European Patent Office, national applications and US Patent and Trademark Office.) Strikingly, the data reveal that 40 per cent of the 652 patents originated from public-sector institutions, mainly US universities and medical charitable foundations. This figure is remarkably high and double the estimate of public-sector patenting activity in the same area between 1984 and 1995 (S. M. Thomas *et al. Nature* **380**, 387–388; 1997).

Only half the patents were filed by the private sector. Approximately 50 per cent of the 157 companies involved were American, 21 per cent Japanese and 18 per cent from Europe (see figure). Despite the huge R&D

spend of the world's multinationals (MNEs), they have been able to claim only 26 per cent of this patent total. The small US biotechnology companies have almost equal impact, accounting for 24 per cent. By contrast, the relatively immature European small and medium-sized enterprises (SMEs) account for only 3 per cent of the total.

The US multinationals have a relatively small stake in these patents. Paradoxically, Merck, with its commitment to public release of human expressed sequence tags, is the exception, having more than any other company. Only 8 per cent of the total belong to US multinationals, a mere third of the US SME fraction. Why are these large companies not more prominent? Many, particularly those in Europe, have strategic alliances with the small US genomics companies where the emphasis may well be on the multinational partner having an



Human DNA sequence patent applications published in 1995 — nationality and sector.

exclusive licence rather than ownership of a patent *per se*. The very recent increased patent filing activities of some multinationals will, moreover, be sufficiently represented in patent databases.

The largest single category is in the area of diagnostics. This is unsurprising as any gene sequence can be used to search for mutations in a particular gene. What is certain is that only a small proportion of these patents will actually be used. These data provide evidence of a realization on the part of public-sector scientists that patenting optimizes the chances of patients receiving benefits from their scientific research. US charities, universities and research institutes alike are filing for patents knowing that industry will not develop new treatments based on inventions without adequate intellectual property protection. We conclude that the increased rates of gene sequencing in a culture that lauds the entrepreneur are stimulating growth in patenting in both the US private and public sectors.

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Keeping a balance

Sir—In all the discussion I have seen of the Dearing report on higher education in the United Kingdom, an important point does not seem to have been made explicitly. For any given level of funding of research, the scientific output will be optimized for some ratio of expenditure on researchers and on their equipment. If too high a proportion is spent on equipment, expensive apparatus will be idle for some of the time; if too high a proportion is spent on scientists, many of them will have obsolete equipment. There is little doubt that this latter situation describes the position in the United Kingdom.

Moreover, if the number of claimants is excessive in relation to the available funding, a great deal of time is wasted in writing and refereeing grant applications that in spite of their good quality cannot be supported.

It is therefore imperative that the size of the claimant pool should be set firmly on a downward course, which does not seem to

have been happening. Such a reduction cannot be quick or painless (or even fair) but, unless this aim can be kept firmly in mind, there must be serious concern about the future health of British science.

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Wisdom in physiology

Sir—The members of the council of the International Union of Physiological Sciences and all speakers at the round table on integrative physiology at the International Congress of Physiological Sciences held in St Petersburg on 3 July unanimously support integrative or systemic physiology. They strongly believe that a new generation of physiologists needs to think at a wider level than that of molecular biology.

I agree with this “call for wisdom”, as Denis Noble of the University of Oxford defined it. In my opinion, biologists are

now becoming more and more like geographers, with a microscope instead of a telescope in their hands.

‘Integrative physiology’ means different things to different people. There are many regulatory systems in physiology, all with specific roles in different organisms. It is essential to find out more about these systems by making the field more popular.

Journals must change their policies, and help to return integrative physiology to the forefront of research. Too many articles are at present returned to authors without peer review. Some, of course, are worthy of that fate, but not all.

There are at least two reasons for hope. First, some laboratories still maintain the old tradition of systemic physiological research; and, second, ‘systemic’ research is far cheaper than ‘molecular’ research, giving more chance to small, independent laboratories and groups.

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Ain't misbehavin'

Sir — The review by Steven Rose of *Behavioral Genetics* by Plomin, DeFries, McClearn and Rutter (*Nature* **388**, 138; 1997) gives a distorted portrayal of the attitudes and intentions of the authors, which is accentuated by the selection of the heading of "Men behaving badly".

I have used earlier versions of this textbook on undergraduate courses and welcomed the production of the third edition earlier this year. One of the authors' summary statements (page 87) states that "Genetic influence on behavior is just that — an influence or contributing factor, not preprogrammed and deterministic. Environmental influences are usually as important as genetic influences." It is difficult to reconcile this statement with Rose's view that the authors have a "genetically deterministic view of human behaviour".

Rose states that the authors give a "quick flip through" basic genetics. The presentation of the biological basis of inheritance and the molecular and quantitative genetic research strategies that are currently employed takes up the first 107 pages of what is a 367-page book — hardly a "quick flip". Moreover, the book is centrally concerned to integrate molecular and quantitative genetic approaches (for example, the opening of Chapter 6) rather than with "quantitative and statistical population genetics" approaches alone, as Rose states.

Rose claims that the textbook ignored the "social context" of human behaviour. One quotation from the introductory section to the chapter in the book devoted to findings and concepts related to environment effects can counter this: "Genetic research will profit if it includes sophisticated measures of the environment, environmental research will benefit from the use of genetic designs, and psychology will be advanced by collaboration between geneticists and environmentalists." The specific issue of the impact of social context on the expressions of genetic effects on individual differences in behaviour is extensively discussed as genotype–environment interactions (six entries in the list of contents).

Rose suggests that a failing in this textbook is the absence of an assessment of psychiatric categories being used. The book makes constant, and deliberate (see page 108), reference to the *Diagnostic and Statistical Manual of Mental Disorders* as one of the standard schemes of classification. *Behavioral Genetics* was not designed as a basic general textbook in either psychiatry or psychology, and the authors can rightly assume that these issues

would be covered elsewhere in a student's course. The actual and potential contribution of genetic studies to the development of nosology are, however, identified and discussed.

The most serious charge made by Rose is that the authors have been "scrupulous with neither data or presentation". The only specific instance of distortion or an alternative interpretation of data mentioned in the review is that the authors have not reflected on whether quantitative trait loci (QTL) are a "biological reality" or a "statistical artefact". In the book there are three main sections on QTL. They are introduced with reference to animal studies, including the studies by Ghosh and colleagues on diabetes in the mouse (*Nature Genet.* **4**, 404–409; 1993). The second is the QTL for reading ability on chromosome 6 (L. R. Cardon *et al.* *Science* **266**, 276–279; 1994). Finally, the QTL associated with drug and alcohol related behaviour in the mouse are discussed (J. C. Crabbe *et al.* *Science* **264**, 1715–1723; 1994). The best protection against statistical artefact in this context is replication and, for the application to human abilities, the authors quote the replication in a second sample by Cardon and in an independent sample by Grigorenko.

Another aspect of partiality according to Rose is that the textbook is "illustrated by pictures of leading advocates in the field, supplemented by 'boxes' of uncritical hagiography". I have reread these boxes carefully in the light of Rose's comments and can find no evidence of inflated or exaggerated claims of the importance of the work of these researchers. There is in fact a striking absence of qualifiers in the description of their work — beyond a "many" when describing the papers they have produced. Despite being somewhat dry, these biographies were, I thought, a valuable addition to this edition.

What I want my psychology students to appreciate from a course in behavioural genetics is how the techniques of both molecular and biometrical genetics can be applied to behaviour, what are the methodological problems that arise in genetically informative research on human behaviour and that it is the joint action of genetic and experiential factors that produces individual differences. With this aim in mind I shall unhesitatingly continue to use *Behavioral Genetics* as a recommended text on my courses and, unlike Rose, can endorse it as the definitive introductory text in this field.

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Faraway Faraday

Sir — Your report that an edited version of last year's Christmas lectures at the Royal Institution (RI) will be presented in Japan is gratifying (*Nature* **388**, 221; 1997).

Abbreviated versions of RI annual lectures (initiated by Michael Faraday in 1826) started in Japan in 1990 and have attracted large numbers of schoolchildren. This is not surprising, as Japanese translations of Faraday's *Chemical History of a Candle* (RI Christmas Lectures 1859–60) have gone through more than 70 editions, and are recommended reading for Japanese schoolchildren.

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Skewed citations

Sir — You report that scientific papers from the United Kingdom have been cited on average 4.19 times but that those from Japan notch up only 3.18 citations (*Nature* **387**, 537; 1997). Do these relative scores reflect more than the reading (and non-reading) habits of Americans, who themselves dominate the writing (and citing) of scientific literature?

A paper's 'impact factor' measures, as much as anything else, how visible and accessible it is in the United States. It would be misleading and unfair to our Japanese colleagues to infer a genuine 'quality gap' from bibliographic data of this kind.

Martin Rees

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Natural name selection

Sir — Butlin and Tregenza¹ quite rightly recognize Dobzhansky's contribution to the debate about whether natural selection can act directly to increase the reproductive isolation of incipient species^{1,2}. We should remember, however, the source of this insight in the work of Alfred Russel Wallace³. Verne Grant has proposed that the phenomenon of reinforcement should be named in his honour the 'Wallace Effect'^{4,5}.

James Murray

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Charlottesville, Virginia 22903, USA

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2. Dobzhansky, Th. *Genetics and the Origin of Species* (Columbia Univ. Press, New York, 1937).

3. Wallace, A. R. *Darwinism* (Macmillan, London, 1889).

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Australian innovation under threat

An apparently successful tax-break scheme to stimulate private-sector investment in research and development has been the victim of illogical cost-cutting. Pressure is building to have it restored.

Ian Lowe

The best way to stimulate the economy is by education and research. That was the unequivocal conclusion of the US government's council of economic advisers in its 1995 report, *Supporting R&D to Promote Economic Growth — The Federal Government's Role*. But research can have no economic impact if the new scientific discoveries are not translated into marketable goods and services.

This has long been a problem in Australia, where the level of scientific research and number of publications per head of the population is respectable. Data from the Institute for Scientific Information in Philadelphia put it about tenth on the Organization for Economic Co-operation and Development (OECD) 'league table', with a publishing rate per head about the same as the United States and the United Kingdom. But Australia's record of translating this academic expertise into commerce is very poor. In 1982, the ratio of exports to imports of technology-based products was 0.12, lower than those for Greece, Turkey, Spain or Portugal.

One of the significant policy initiatives of the Australian Labor government elected in 1983 was a 150 per cent tax concession for private-sector research and development (R&D). This had a dramatic effect. Private R&D trebled in the next 10 years. There was some scepticism at the time about whether the effect was real. There were worries about 'creative' accounting, and suspicions that, as the tax break gave an incentive to measure and declare the level of R&D spending, the apparent increase could simply be a reporting effect. But most early doubts have been allayed by independent measures that have shown, for example, a significant improvement in levels of overseas patenting and receipts from sales of technical knowledge.

The figures are startling: private-sector R&D had risen from 0.24 per cent of gross domestic product in 1982 to 0.74 per cent in 1994. The rate of growth since 1988 has averaged 8 per cent per year, compared with 1.6 per cent in Japan, 0.6 per cent in the United States and -0.2 per cent in the United Kingdom. External patent applications per unit of economic output more than doubled during the 1980s, whereas the OECD average increase was about 50 per cent. Australian applications for patents in other countries grew from about 1 per cent of the OECD figure in 1981 to about 3 per cent in 1993. The growth in patenting activity correlates remarkably well with the growth in business

R&D for the decade 1983-93. And the ratio of sales to purchases of technical knowledge increased during the 1980s from an embarrassing 1:8 to a respectable 1:3.

All the signs indicate that the tax incentive has been associated with a dramatic rise in private-sector innovation. One example is Solarhart, a small West Australian company which is a local brand leader in domestic solar-water heaters. Solarhart embarked on a programme of research to improve the quality of its product, resulting in a new design that carries a 12-year warranty, compared with the industry standard of five years. According to the general manager, the company could not have funded the development programme without the tax concession. Other examples of successful products and improvements range from zinc-bromide batteries to new light metal alloys.

Unfortunately, two policy changes have reduced the impact of the tax concession. First, the Labor government lowered the corporate tax rate in 1989, reducing the real value of the tax break to companies. Then the new Conservative government's budget in May 1997 cut the concession to 125 per cent (see *Nature* 387, 222; 1997). The combined effect of these two changes has been to double the real cost of R&D to the private sector.

In its budget statement in May, the government said that the success of private-sector research shows that the concession has done its job and is no longer needed. I cannot see the logic of this argument. All the evidence seems to indicate that the tax concession has been a crucial factor driving the increasing level of business investment in research.

Two recent reports have been implicitly critical of the change. Australia's chief scientist, John Stocker, said in his June review of science that the incentive for business research needed to be increased. And last month's review of industry policy by business executive David Mortimer recommended a new structure equivalent to a tax concession of 139 per cent, a compromise between the current figure of 125 per cent and the old 150 per cent (see *Nature* 388, 8 & 509; 1997).

All the evidence seems to indicate that the tax concession has been a crucial factor driving the increasing level of business investment in research

In my view, a more rational approach would have been to identify and discontinue policies that were not working, while building on successful initiatives. The reduction of the R&D tax concession set new standards for irrational policy: a system that seemed to be working was singled out for a cut.

In its 1995-96 budget statement, the government said that it could achieve its objectives for innovation "through greater use of targeted outlay measures which are more transparent, less open to abuse and will not impose uncontrollable contingent liabilities on the budget". But no evidence has been presented to support the assertion that the tax concession was open to abuse.

I could understand the justification for targeted cutbacks, for example restricting the tax break to, say, emerging high-technology industries. But I think that the real problem was that Treasury officials in a new administration were nervous about what appeared to them to be a blank cheque, a liability of unknown (and unknowable) proportions. But by making a blanket cut, the change in policy does not solve the problem — it simply makes the unknown liability smaller.

What policies should be used? In my opinion the 150 per cent tax concession should be increased to 200 per cent, rather than be cut back. Singapore, whose government recognises the economic value of innovation, currently offers a 200 per cent concession. It is a good approach for any government to retain the principle that the best people to decide the level and direction of spending on innovation are the private corporations, and to offer an attractive incentive to them to proceed.

Each government determines how its country can compete internationally, through taxes, subsidies, tariffs and other measures. These arrangements inevitably influence companies' investment decisions.

If governments have innovation as their main objective, they need to make concessions accordingly. The tax concession for private-sector R&D was a good example of sensible, successful positioning of Australia in global terms, because it influenced the way companies behaved. The concession should be restored to its previous value in real terms. Otherwise, the only sort of economic activities encouraged by Australia's incentive structure are mining, land speculation and corporate asset-stripping. It should come as no surprise if these enterprises flourish while innovation languishes. □

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The right sort of roughness

Peter Carpenter

Skin-friction drag affects any surface moving through a fluid. But a new theory of near-wall turbulence has led to a strange new drag-reduction technique: applying a random arrangement of bumps.

Every surfer believes that polished boards create less drag. This view has always been somewhat at odds with the natural world, where smooth surfaces are rare. It was further undermined with the discovery by the US space agency NASA¹, and by scientists studying the hydrodynamic effects of shark scales², that surfaces with riblets — minute streamwise grooves — experience lower drag than smooth ones. The 'smooth is best' precept has now received a further setback. On page 753 of this issue³, Sirovich and Karlsson show that surfaces covered with special randomized patterns of small v-shaped protuberances produce lower drag than smooth ones. But regular patterns of the same protuberances lead to a rise in drag, so the surfers are not completely wrong!

The type of drag in question is skin-friction drag, which is caused by the action of viscous shear forces on the surface. It is much greater when the flow is turbulent, as in most engineering applications. Drag reduction has been an obsession of practical aerodynamicists for most of the twentieth century; equally, achieving a theoretical understanding of turbulence has remained the elusive goal of many of the finest minds during the same period. Hitherto, the discovery of new drag-reduction methods has done far more to advance the theories of turbulence than vice versa. In this respect Sirovich and Karlsson are a rare exception, because their drag-reduction method is the fruit of their efforts to develop a theoretical understanding of the turbulent air flow in a two-dimensional channel.

The turbulent flow near a channel wall is bewilderingly complex. This complexity is inherent in the flow physics, and stems, fundamentally, from Newton's laws of motion. Turbulence is found when the effects of viscosity are weak — it is an intriguing paradox of fluid mechanics that the weaker the direct action of viscosity the more complex its effects. Most authorities now agree that deterministic structures (usually termed coherent structures), which are predictable in their general form, are created in turbulent flow. But these coherent structures have a broad, random distribution in time and space, and they are characterized by a very wide range of length and timescales.

It is generally believed that the crucial dynamics occur in a thin, highly sheared,

region, extending up to 2 mm from the wall in the channel-flow experiment of Sirovich and Karlsson. A veritable zoo of coherent structures has been identified by successive experimentalists, and a common view of the dynamics has emerged (Fig.1). Low-speed streaks appear near the wall⁴, caused by streamwise vortices. As these vortices develop they lift up away from the surface ('ejection') and finally 'burst'. Bursting generates intense turbulence and appears to initiate the formation of new vortex structures.

The key questions are, what causes ejection and bursting, and how does bursting create new vortices? There are many different theoretical models. For example, Smith *et al.*⁵ believe that the bursts occur when a vortex approaches the wall, causing strong local flow deceleration and subsequent flow separation (eruption) from the wall, thereby triggering the birth of a new vortex. On the other hand, Sirovich and his co-workers believe that, although the vortices are the primary structures, there are also much weaker oblique plane waves propagating downstream, and that bursting and rebirth come about through an interaction between a pair of waves and the vortices. In previous computer simulations they found that artificially randomizing the phases of the vortices and oblique waves led to less bursting and consequently to a reduction in drag.

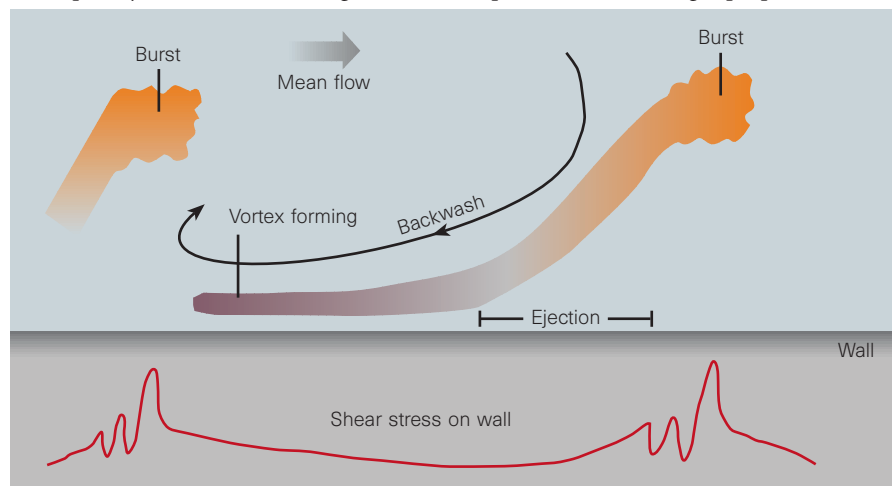


Figure 1 Simplified side view of near-wall turbulence. Most of the shear stress (or drag) is created where vortices 'burst' away from the wall. An unproved idea is that bursting is caused by an interaction between the vortices and pairs of oblique plane waves. It has led to an effective new method of drag reduction³: randomized bumps that break up the phase coherence of the vortices.

The authors attempted to mimic this effect in the experiments by randomizing the arrangement of the v-shaped protuberances. In this way up to 12 per cent drag reduction was obtained, accompanied by reduced bursting frequency, in line with the numerical experiments. But regular arrays of the same protuberances led to a substantial drag rise.

Oddly, the maximum drag reduction achieved with conventional riblets is also about 12 per cent. But riblets are thought to make the vortices more ordered — it would be difficult to explain their effect in terms of phase randomization. Equally, it is difficult to explain the effects of phase randomization in terms of the other theoretical models. The secrets of turbulence appear to be as elusive as ever.

The near-wall turbulence for aircraft wings is quite similar to channel flow. So, would this new technique be practical for aircraft? For a large subsonic airliner in cruising flight, the near-wall region is less than 200 μm thick, owing to the much greater flow speed. Accordingly, this is roughly the required span of an individual protuberance. This may seem very small, but it is about ten times the spacing of the riblets that have been successfully flight-tested.

However, skin friction only contributes 30 per cent of the total drag for a typical subsonic airliner. (The other two main sources of drag are lift generation and shock waves.) So the randomized protuberances could lead to an overall drag reduction of about 3 per cent. In terms of the total amount of fuel used by the world's airlines in one year, this would still constitute a very large saving, but it only represents about 0.3 per cent of the running costs of an individual aircraft. To be acceptable to an airline, a drag-reduction method must not increase the aircraft's weight (as this causes a proportionate rise in drag), increase the capital cost appreciably (as this represents a much larger proportion of the

running costs than fuel), or interfere with maintenance, either compromising safety or increasing costs.

For these reasons, riblets have not yet been used for commercial aircraft; and the new method would probably be no more attractive. However, it may have advantages for other engineering applications, for example high-speed sailing yachts, such as America's Cup competitors, for which skin friction contributes more than half the total drag. □

Apoptosis

CED-4 is a stranger no more

Michael O. Hengartner

The nematode worm *Caenorhabditis elegans* has been used with great success to identify the basic components of the machinery underlying apoptosis (programmed cell death)¹. Indeed, of the three key cell death genes that have been identified in *C. elegans*, two — *ced-3* and *ced-9* — have mammalian homologues that also function in apoptosis. But the sequence of the third gene, *ced-4*, revealed no obvious mammalian homologue, and precious little in terms of possible mechanism of action. A flurry of activity has changed that. A paper by Zou *et al.*, published earlier this month in *Cell*², provides a homologue. And work by Chinnaiyan *et al.* (page 728 of this issue³) and by Seshagiri and Miller in *Current Biology*⁴ lays down some choreography for the part that CED-4 protein plays in the molecular dance of death.

Over the years, genetic screens in *C. elegans* have led to the identification of about a dozen cell death (*ced*) genes that are responsible for one aspect or another of the apoptotic process. Three of these genes stand out. Two, *ced-3* and *ced-4*, are essential for cell death. The third, *ced-9*, antagonizes the proapoptotic activities of *ced-3* and *ced-4*, and thereby protects cells that should survive from any accidental activation of the death programme.

Real stardom for *ced-9* and *ced-3* only came with their cloning, when it became clear that they encode components of a universal and highly conserved death machinery: CED-9 protein turned out to be a member of the Bcl-2 family of cell death regulators, whereas CED-3 was homologous to a family of proapoptotic cysteine proteases, known as the caspases. Indeed, identification of CED-3 as a caspase homologue was the first evidence of this family's involvement in apoptosis. In both worm and human, CED-9/Bcl-2 act upstream of the 'execution caspases' such as CED-3 and caspase-3, somehow preventing their proteolytic processing from zymogens into fully active killer enzymes. But how Bcl-2 *et al.* perform this feat remains very much up in the air.

The *ced-4* story was initially much less glamorous. Because the gene encoded a pre-

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viously unknown protein without any mammalian homologue, its cloning did not provide much help in explaining how it might promote cell death. Genetically, *ced-4* had been placed between *ced-9* and *ced-3* in the pathway leading to cell death⁵, suggesting that it might act as an adaptor, linking the upstream regulator CED-9 to the downstream death effector CED-3.

Strong experimental support for this view came from four papers published early this year, which collectively showed that CED-4 can interact directly and simultaneously with both CED-9 and CED-3 (reviewed in refs 6, 7); the interaction must be quite stable, as CED-4 and CED-9 colocalize when co-expressed. These observations point to a model in which CED-9 prevents cell death by directly binding to a CED-4/CED-3 complex, presumably keeping it in

an inactive conformation. Upon reception of a death-inducing stimulus, the complex (already dubbed the 'apoptosome' by pundits) might dissociate, freeing CED-4/CED-3 to get to work (Fig. 1a).

But being a linker between CED-9 and CED-3 cannot be the whole story: the fact that CED-4 is essential for cell death in *C. elegans* indicates that it must have a specific proapoptotic activity. The two papers by Chinnaiyan *et al.*³ and Seshagiri and Miller⁴ describe just such an activity: both groups found that processing of proCED-3 into the active protease was dramatically increased in cells that also expressed CED-4. This stimulation was inhibited by mutations known to knock out the death-promoting activity of CED-4, or by co-expression of wild-type (but not mutant) CED-9. These observations fit very nicely with the apoptosome theory, and also explain why CED-3-mediated killing is so inefficient in the absence of CED-4.

How does CED-4 promote CED-3 activation? One possibility is that CED-4 is itself a protease that can cleave proCED-3. Arguing against this hypothesis, Chinnaiyan *et al.*³ showed that processing of an enzymatically inactive CED-3 mutant is not stimulated by CED-4. Thus, the obvious alternative is that CED-4 promotes CED-3 autoprocessing. A hint of how it might do so comes from the observation that CED-4 contains a predicted nucleotide binding site, which seems to be functional because point mutations in the site abolish the proapoptotic activity of CED-4 in a number of experimental systems^{3,4,8}.

Furthermore, Chinnaiyan *et al.*³ have now shown that purified wild-type CED-4

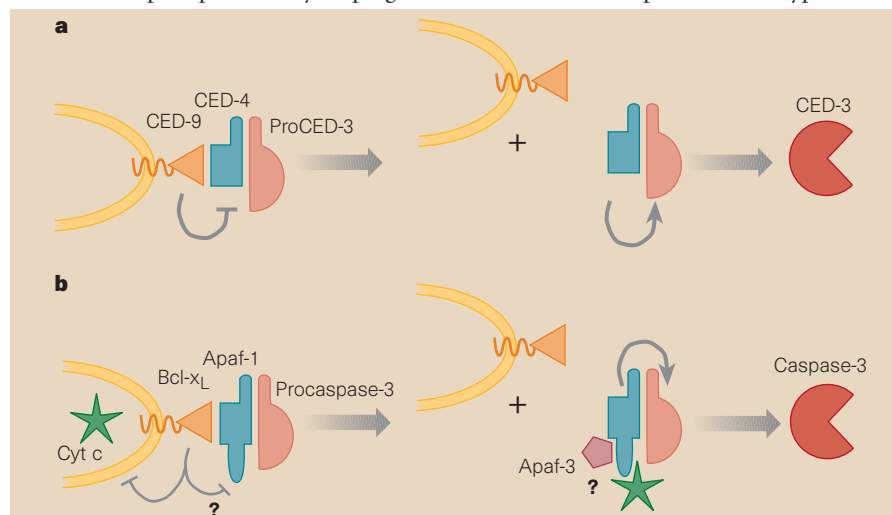


Figure 1 Caspases are cysteine proteases that promote apoptosis in mammals and have homologues in *C. elegans*. a and b depict highly simplified schemes of how they might be activated in the two systems. a, In *C. elegans* the inactive CED-9/4/3 complex is bound to cell membranes by CED-9's hydrophobic tail. On reception of a death-inducing stimulus, something happens (here shown as the physical separation of CED-4/3 from CED-9) which allows processing and activation of the 'execution caspase' CED-3. b, In mammals, the Bcl-2 family might perform double duty, preventing cytochrome c from leaving mitochondria and also possibly binding to Apaf-1 (one of the so-called apoptosis protease-activating factors, of which cytochrome c is Apaf-2). The death-inducing stimulus would allow formation of the full Apaf complex, possibly including Apaf-3, leading to caspase-3 processing and activation. Question marks point to aspects that are even more egregiously speculative than the rest of the model.

(but not a deletion mutant lacking the ATP binding site) can specifically bind to ATP analogues. Although nobody has yet shown that ATP hydrolysis occurs, it seems likely that CED-4 acts as a context-dependent ATPase. By analogy with the heat shock proteins, it could be that CED-4 is a proCED-3-specific chaperone, stabilizing and regulating the inactive proform. ATP hydrolysis, which might normally be inhibited by CED-9 binding, could provide the energy necessary for a conformational change and autocatalytic activation of CED-3.

Is any of this information relevant to caspase activation in mammals? The first hint that it is came from the observation by Chinnaiyan *et al.*⁹ that Bcl-x_L will readily associate with caspase-1 when the two proteins are co-expressed in mammalian cells, even though they show no affinity for each other *in vitro*. The authors suggested that an endogenous, CED-4-like activity (and thus a mammalian apoptosome) exists in mammals that could act as a bridge between Bcl-x_L and caspase-1.

Even more dramatic evidence for a worm-mammal connection has been unearthed by Zou *et al.*². This group had previously¹⁰ developed an extract system derived from human cells, that, upon the addition of deoxyribose-ATP, would result in the proteolytic activation of caspase-3. Purification of the components in the extract required for this activity led to the isolation of three apoptosis protease-activating factors (Apafs), which in combination seem to be sufficient to promote caspase-3 processing upon addition of deoxyribose-ATP. So, at least functionally, the Apafs are the mammalian equivalent of *C. elegans* CED-4. Rather surprisingly, Apaf-2 is cytochrome *c*, the ubiquitous protein involved in the mitochondrial electron transport chain¹⁰. The counterintuitive corollary of this observation is that, in cells doomed to die, cytochrome *c* must leave the mitochondria for the cytosol (where the caspases reside). Counterintuitive this may be, but it appears to be true, at least in mammalian cells (reviewed in refs 5, 11).

In their paper, Zou *et al.*² present the sequence of Apaf-1, a protein of *M_r* 130,000. Remarkably, part of it shows a striking similarity to that of CED-4, with the two proteins aligning over most of the CED-4 sequence, including the nucleotide binding site. Flanking the CED-4 homology region are two other domains that provide additional hints as to how the Apafs might direct caspase activation (Fig. 1b). The amino terminus of Apaf-1 shows some sequence similarity to the prodomain of CED-3, and probably constitutes a caspase-recruitment (CARD) domain. CARD domains are found in a number of cell death proteins (including CED-3 and CED-4) and may bind directly to caspases¹². This domain might thus be the link between the Apafs and caspase-3. At the

carboxy terminus of the protein is a large domain containing 12 WD-40 repeats. Such repeats usually mediate protein-protein interactions, and could in this case be involved in the physical interaction that Zou *et al.*² observed between Apaf-1 and Apaf-2/cytochrome *c*.

So what does it all mean? That both CED-4 and Apaf-1 promote the activation of caspases suggests that their molecular mechanism of action is likely to be the same. However, as is the case with CED-9 versus Bcl-2, and CED-3 versus the caspases, regulation of Apaf-1 is likely to be more complex (or sophisticated, depending on your point of view), not least because mammalian cells must integrate many more signals before deciding whether to live or die.

Plenty of issues remain. Is Apaf-1, like CED-4, regulated by interaction with Bcl-2 family members? If so, does it show any preferences in its dealings with the various factions within the family? What are we to make of the WD-40 repeats in Apaf-1, and of their absence in CED-4? If they bind to cytochrome *c* (and possibly Apaf-3), does binding result in activation, or in relief of an intrinsic inhibitory activity? And are we to conclude that, in *C. elegans*, cytochrome *c* is not involved in cell death?

Finally, does Apaf-1 constitute the only

mechanism that leads to caspase-3 activation? In *C. elegans*, this is clearly the case, because in the absence of CED-4 there is no cell death whatsoever. Such a dramatic effect seems unlikely in mammals, be it because of alternative activation pathways or redundancy with other caspases. Indeed, what about the other caspases? Do they each have their own Apaf-1 homologue, do they share a common activator, or are they simply activated by other caspases (through the fabled caspase cascade)? Questions, questions — much work remains to be done. □

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Microporous solids

Cobalt caged for catalysis

Robert L. Bedard

On page 735 of this issue¹, Stucky *et al.* introduce a new method of making microporous materials that contain transition metals. They demonstrate it by synthesizing cobalt phosphates and cobalt metal phosphates, and the family of materials that can be made in his way should include a large variety of compositions and crystalline structures, with the potential for new catalytic reactions

In most cases, the new cobalt phosphate structures have been observed before in the aluminosilicate zeolites, a class of materials that are not only synthesized industrially as catalysts and adsorbents, but also formed in nature by the weathering of volcanic rock and marine sediments. Similar structural mimicry of these naturally occurring minerals has been observed before in beryllium² and zinc³ phosphate frameworks, but its extension into the transition elements is of interest because of their greater variety of useful chemical properties. With many oxidation states so easily accessible, transition metals will allow a wider range of reactions than the solid-acid chemistry of traditional zeolites — they should be able to catalyse oxidation and reduction reactions for example. The magnetic behaviour of the transition

metals might also be exploited to create new types of molecule-selective devices.

The commercial applications of microporous materials are continually expanding. Materials of this type are often referred to as frameworks, to describe their scaffold-like, rigid crystalline structures; or as molecular sieves, because their pore cross-sections are of molecular dimensions. As well as the aluminosilicate zeolites, there are titanium silicates, aluminium phosphates, metal sulphides and selenides, transition-metal oxides, and many others.

These materials are used as catalysts in refining gasoline and diesel fuel; they aid powdered laundry detergents by softening water; and they can efficiently separate air into its valuable pure components, N₂ and O₂. Newer applications include the use of the molecule-sized cavities within these materials to carry out the selective oxidation of simple organic compounds into useful chemicals⁴. Microporous oxides, used as ion exchangers to selectively trap the highly radioactive isotopes ¹³⁷Cs and ⁹⁰Sr, are likely to play a leading role in cleaning up the nuclear waste generated by weapons programmes and nuclear power generation^{5,6}.

The challenge in synthesizing new frame-

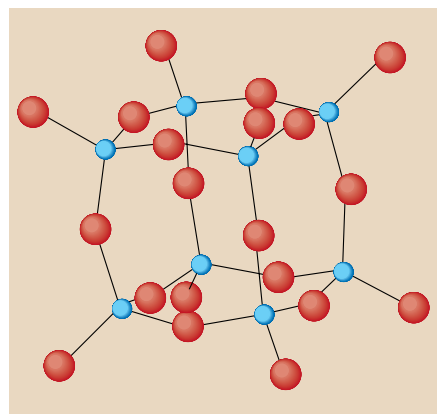


Figure 1 This hypothetical structure was proposed for an aluminosilicate zeolite⁷, but has only now been realized as a cobalt phosphate. The new material has cobalt, aluminium and phosphorus at the blue sites, and oxygen at the red sites. The method of synthesis should yield a great number of new microporous materials, opening up a wider range of chemistry for these open framework catalysts.

works is to find a recipe that at least partially dissolves and transports the components in the reaction solvent, and also causes a microporous framework to form rather than an undesirable non-porous structure. The significance of the new phases¹ lies in their open structures (including one novel form) and their compositions, and in the discovery of a new synthesis procedure that made these materials possible.

The authors chose a clever combination of a new cobalt reagent, CoCO_3 , and a largely non-aqueous ethylene glycol solvent system to create conditions that lead to the ready synthesis of the cobalt phosphates. The pores within the crystallized products are filled with organic ammonium cations and water, which stabilize the structure during crystallization by space filling and charge compensation. But whether this metal carbonate route is more generally applicable to other divalent transition metals is not yet clear.

Another intriguing aspect of the cobalt

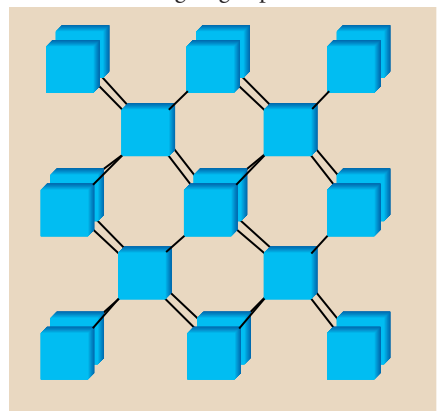


Figure 2 Cubic framework of the building block in Fig. 1. The pseudo-octagonal micropores in this structure are 3.8 Å in diameter and permeate the structure in three dimensions.

phosphates is the formation of an unprecedented structure that Stucky and colleagues have called ACP-1, for aluminium cobalt phosphate 1 (Fig. 1). This structure was erroneously proposed for one of the synthetic aluminosilicate zeolites, known as cubic zeolite P, by R. M. Barrer *et al.*⁷, in 1959. Nearly 40 years have passed between Barrer's proposal and the successful synthesis of the hypothetical structure, albeit in a completely different composition. The ACP-1 structure is an array of nine cube-shaped building blocks arranged at the corners and in the centre of a larger cube. Repeating this structure produces a framework that contains a three-dimensional network of 3.8-Å channels (Fig. 2).

A key question that always arises when a new family of frameworks is discovered is whether the latent microporosity of the materials can be accessed. The porosity remains latent until a method is devised to remove the space-filling, charge-compensation molecules without destroy-

ing the framework. Without the removal of this 'stuffing', much of the promise of novel or improved applications is diminished. Achieving access is a crucial step in the application of the cobalt phosphates — if successful it will allow study of the redox chemistry of the frameworks and of their potential as selective oxidation catalysts. The cobalt phosphates may also be good at adsorptive separations of gases and ion exchange processes. □

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Neurodegeneration

A silent channel opens its gates

Peter H. Seeburg

Many human neurodegenerative diseases, such as amyotrophic lateral sclerosis (also known as Lou Gehrig's disease), Alzheimer's disease, Parkinson's disease and Huntington's chorea, lead to the demise of distinct nerve-cell populations. Yet in no case is the selective vulnerability of the affected cells, nor the underlying damaging principle, understood.

A popular view is that these sinister diseases are caused — or at the very least exacerbated — by the unphysiological activity of cation-selective membrane channels operated by glutamate, which is the most abundant excitatory neurotransmitter in the brain. Now, the discovery by Zuo *et al.*¹ (page 769 of this issue) provides the first direct evidence in support of this view. The authors have traced the selective neurodegeneration that occurs in a spontaneous neurological mouse mutant (*lurcher*) to the constitutive activation of a member of the glutamate-receptor-channel family.

First described in 1960, the *lurcher* (*Lc*) mouse derives its name from a peculiar gait that is characteristic of heterozygous carriers of the mutant gene. These mice develop a lack of balance (ataxia), because all of the Purkinje cells in the cerebellum — a brain structure that is important for motor coordination — are lost. Mice that are homozygous for the *Lc* gene die shortly after birth, due to a massive loss of hindbrain neurons during embryonal development. These neurons include the trigeminal motor nucleus, which controls the muscles required for suckling. So, the neurological phenotype of the *lurcher* gene is characterized by selective neuronal

cell death. In a collaborative effort¹ between the labs of Nathaniel Heintz and David J. Linden, the molecular cause for this neuronal death has now been unravelled.

Ever since John Olney and Lawrence Sharpe² reported that infant rhesus monkeys develop brain lesions when exposed to glutamate, attention has been directed towards how the abundant excitatory neurotransmitter L-glutamate might become a culprit in acute and degenerative cell death in the brain. In an elegant series of experiments, Dennis Choi³ and colleagues showed that this glutamate-evoked cell death can be mimicked *in vitro*, and that the onset and extent of cell death are critically dependent on the influx of Ca^{2+} into neurons.

Our mechanistic understanding of these observations was boosted by the molecular characterization of the various receptor channels that are activated by L-glutamate in central neurons⁴. These cation-selective channels are composed of sequence-related subunits. They form a family of glutamate receptors (GluRs) which have distinct properties, commensurate with their particular roles in synaptic physiology. The best-characterized GluRs are the AMPA (amino-hydroxymethylisoxalate propionic acid) and NMDA (*N*-methyl-D-aspartate) receptor channels, which mediate fast excitatory neurotransmission.

Whereas most AMPA receptors are permeable only to monovalent cations, NMDA receptors are designed for the controlled synaptic influx of Ca^{2+} ions. Most neurons carry both types of GluR, not only in synapses

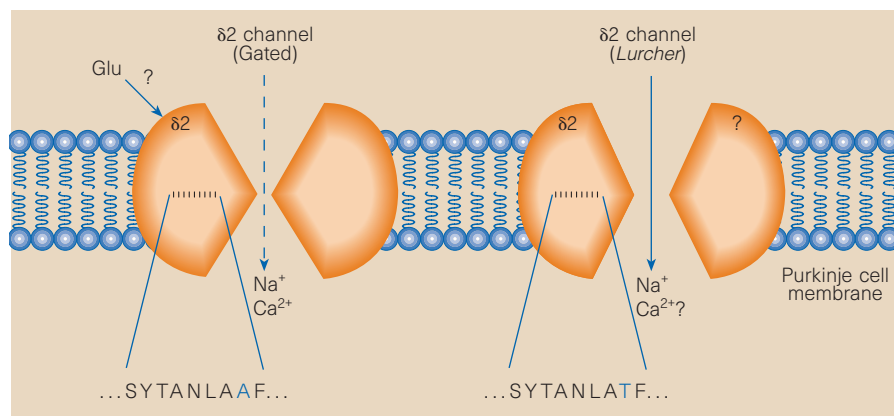


Figure 1 Purkinje cells in the cerebellum express the glutamate receptor (GluR) $\delta 2$ subunit which, possibly together with an unknown subunit partner, forms a cation channel that is activated by glutamate or by a related excitatory transmitter. The gated channel is permeable to Na^+ and it may also be permeable to Ca^{2+} ions. Zuo *et al.*¹ have found that, in the *lurcher* mouse, an amino-acid substitution in a short sequence that is conserved in all GluR subunits (dotted line in $\delta 2$, magnified to show the actual sequence) activates the $\delta 2$ channel in the absence of neurotransmitter, leading to neurodegeneration of Purkinje cells.

but also on dendrites and cell bodies. This explains why the cell death that is encountered in acute neurological insults such as stroke and head trauma is delayed: injured cells release glutamate which opens the gates of AMPA- and NMDA-receptor channels. This leads to an ultimately deadly influx of Ca^{2+} to the neurons that surround the main site of injury.

Experimental evidence implicating glutamate in acute neuronal cell death came easily (for example, refs 2, 3). However, a link between neurodegenerative diseases and genetically determined alterations in signalling cascades operated by glutamate or other excitatory neurotransmitters has not yet been forged. Enter $\delta 2$ — a lesser-known member of the GluR family. From the molecular signature of $\delta 2$ we might predict that it is operated by glutamate, even though the recombinantly expressed protein lacks all channel activity^{5,6}. Expression of $\delta 2$ seems to be confined to a subset of dendritic spines⁷ in cerebellar Purkinje cells, and knock-out of the $\delta 2$ gene leads to aberrant innervation and altered synaptic physiology of these large, cerebellar neurons⁸.

Zuo *et al.*¹ have completed a technical *tour de force* by positionally cloning the *Lc* gene. On doing this, they saw that what they had cloned was the $\delta 2$ gene, carrying a single nucleotide substitution (Fig. 1). This nucleotide change indicated the substitution of an amino-acid residue in a small region that is highly conserved between all known GluR subunits. When the authors expressed the mutant $\delta 2$ subunit *in vitro*, they found that the channel was active in the absence of glutamate — in other words, the mutation had resulted in a gain of function. Indeed, Purkinje cells in heterozygous *Lc* mice show a resting membrane potential that is well above that of their wild-type counterparts, indicating that the affected cells experience a constant influx of cations. So the death of these cells is closely related to

the excitotoxic cell death that is brought about by excess glutamate³.

What have we learned from these findings and what remains enigmatic? We finally have a demonstration that a molecular alteration in a transmitter-gated channel can cause the degeneration of the neurons that express it. The consequence of the single amino-acid change indicates that the highly conserved region in which it occurs is a major determinant in channel activation, following the binding of a neurotransmitter. We do not know the subunit composition of the native channel that incorporates $\delta 2$, how this channel operates normally, or which ligand — glutamate or a related transmitter — gates it.

It remains to be seen whether the channel is also permeable to Ca^{2+} ions. Perhaps the entry of Ca^{2+} into Purkinje cells that are heterozygous for the *Lc* gene is due to depolarization, mediated by the entry of Na^+ through the constitutively open channel. Furthermore, although expression of $\delta 2$ has been documented in Purkinje cells, the massive neuronal loss in the mid- and hindbrain of homozygous mouse embryos indicates that $\delta 2$ is also expressed in these structures — at least transiently during embryonal development. Finally, the finding by Zuo *et al.*¹ should now give considerable impetus to screening for other GluR mutations in familial neurodegenerative disorders. □

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NATURE

100 YEARS AGO

An interesting memoir, by C. T. Mörner, has recently appeared in the *Zeitschrift für physiologische Chemie*, dealing with a method of preserving fish, much employed in northern Sweden. The fish are washed, and placed in wooden casks, and are then covered with brine. The casks are then closed and made airtight, and placed in the open air in a sunny place, and allowed to remain there for from five to six weeks. The process of fermentation, which soon ensues, is controlled by means of a small vent-hole, which is opened from time to time.... As soon as the requisite stage in the process has been reached, the casks are opened, and the now-finished article is packed in smaller vessels for storage and distribution. This article of diet, known in Swedish as "surfsk," is eaten either raw or toasted.... As accounting for the disagreeable odour which characterises this preparation, Mörner found amongst the gases emitted during fermentation the offensive-smelling methylmercaptan. Amongst the organic acids discovered in the "surfsk," whilst absent in the fresh fish, succinic acid, butyric acid, formic, acetic, and valeric acids were detected.... Curiously indol, skatol, phenol, putrescine and cadaverine, so characteristic of putrefactive processes in general, were absent in this preparation. From *Nature* 19 August 1897.

50 YEARS AGO

The Seventeenth International Physiological Congress was held in Oxford during July 21–25. It was attended by about 1,200 physiologists, including welcome delegates from the USSR and China.... Perhaps the most important work reported has been in independent research, in which, after Prof. E. G. T. Liddell's phrase, after seven years, members were showing an active post-inhibitory rebound. An outstanding paper was given by A. L. Hodgkin and A. F. Huxley, who suggested that during the rising phase of the action potential in nerve, the membrane becomes highly permeable to sodium ions which then enter the cell. Potassium ions leave the cell during the falling phase and later restorative processes occur linked with energy-producing mechanisms. There was considerable indirect evidence in favour of this view. From *Nature* 23 August 1947.

Atomic physics

Shaping atoms in optical lattices

Christopher Monroe

Imagine a bowl half full of water. Suddenly move the bowl slightly to the side, and a water wave rocks back and forth on the surface, largely retaining its shape. Suddenly squeeze the bowl down in size, and the surface wave 'breathes' in and out, approaching a sharp point when the wave reaches the centre. Quantum mechanics tells us that individual atoms are also waves, so it shouldn't be a surprise that atom wavepackets might be prepared and manipulated in a similar fashion — even though quantum atom wavepackets are not just ordinary waves; they describe in some sense the atom's existence.

It's a rare physical system that allows such control of individual atoms, but now both breathing and rocking motions have been produced, and observed, in optical lattices. By confining individual atoms in an

array of sites known as an optical lattice, researchers^{1–3} have observed the breathing motion of atomic wavepackets when the atoms were squeezed. The rocking motion of atoms in optical lattices was first observed⁴ in 1996. This shaping of atom wavepackets is of considerable interest to fundamental studies of quantum mechanics, and may also have future applications in nanometre-scale atomic lithography.

Optical lattices are formed by particular geometries of crossed laser beams, which interfere to produce alternating dark and bright spots of light about an optical wavelength apart. Under certain conditions, atoms are attracted to the bright spots due to electric dipole forces, resulting in a lattice of tiny atom traps — like a microscopic egg carton for atoms (Fig. 1). This remarkable form of matter resembles a solid-state crystal,

except that the periodic confining force is under precise external control, allowing some textbook solid-state phenomena to be cleanly observed⁵. In the above experiments, for instance, atoms were squeezed by suddenly increasing the power of the laser beams — thereby deepening the egg cartons. Because the atom wavepackets can't respond fast enough to the change, they pulsate in and out, intermittently occupying a much smaller region of space than they started out with.

Groups at the Max Planck Institute for Quantum Optics in Germany¹ and the US National Institute of Standards and Technology (NIST)² detected this breathing motion by observing the diffraction of a probe laser beam aimed at optical lattices of rubidium and caesium atoms, respectively. Even though the weak probe scatters very little from each individual atom, at certain angles the scattered light interferes constructively, owing to the periodic spatial order of the million or so atoms across the lattice (this effect is similar to diffraction from a grating).

If the atoms at each lattice site are then smeared out, the efficiency of the interfer-

Galactic structure

Bar serves cosmic scorpion

An ultraviolet image of the spiral galaxy, NGC4303 (below), has revealed a remarkable ribbon of star-formation activity that winds all the way into the central hub of the galaxy. But there is a twist to this observation in more ways than one, for it may yield clues to a suspected link between the structure of galaxies and the luminosity of their cores.

This striking image, obtained using the Hubble Space Telescope, was published last month (L. Colina *et al.* *Astrophys. J.* 484, L41–L45; 1997). Resembling a giant scorpion, with the galactic nucleus as its sting, the tapering

spiral structure consists of clumpy regions of bright emission — clear indicators of hot, newly formed stars.

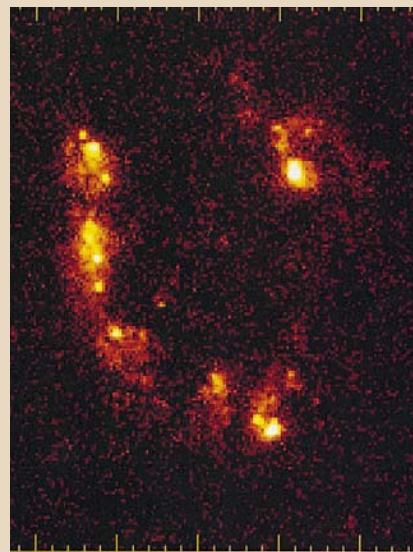
NGC4303 is a barred spiral galaxy. The term derives from a linear grouping of stars, the 'bar', from which the curved arms are observed to emanate. Theoretical simulations suggest that the bars in spiral galaxies may serve a particular function, in gravitationally directing the flow of gas from the outer to the inner nuclear regions.

But the consequences of this gas flow can vary, depending on whether the galaxy's mass distribution gives rise to especially stable orbits, known as inner Lindblad resonances. For example, if such resonances exist, gas will accrue in their vicinity, resulting in the formation of a ring-shaped region of rapid star formation (or starburst) around the galactic centre. But in the absence of these resonant orbits, the bar of the galaxy can in principle direct the gas stream all the way into the nucleus.

No prizes, then, for guessing which of these cases fits NGC4303. Still more encouraging for this picture is the fact that a Hubble image of a second barred spiral galaxy, NGC3351 (above right), demonstrates the alternative ring-like morphology.

But what does this have to do with the brightness of different galactic nuclei?

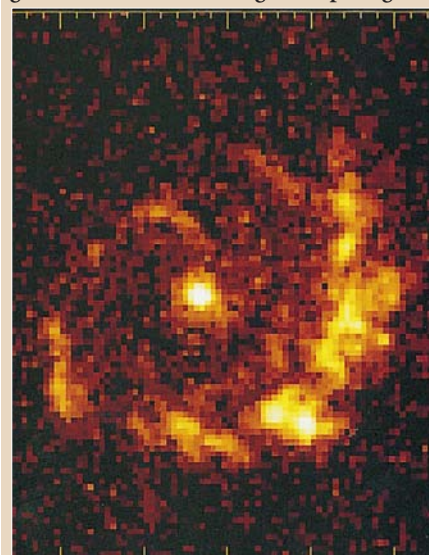
Crucially, NGC4303 and NGC3351 are markedly different in their central regions.



NGC4303 has a luminous core, which is extremely compact by astronomical standards — at no more than about 25 light years across, its inner structural details remain invisible even to the Hubble Space Telescope. By contrast, there is no evidence for an active nucleus in NGC3351 at all.

So it seems likely that the spiral flow in NGC4303 is directly connected with the observed nuclear activity, either feeding a massive central black hole or fuelling an extremely bright cluster of massive young stars, similar to those seen throughout the spiral arm.

Karen Southwell

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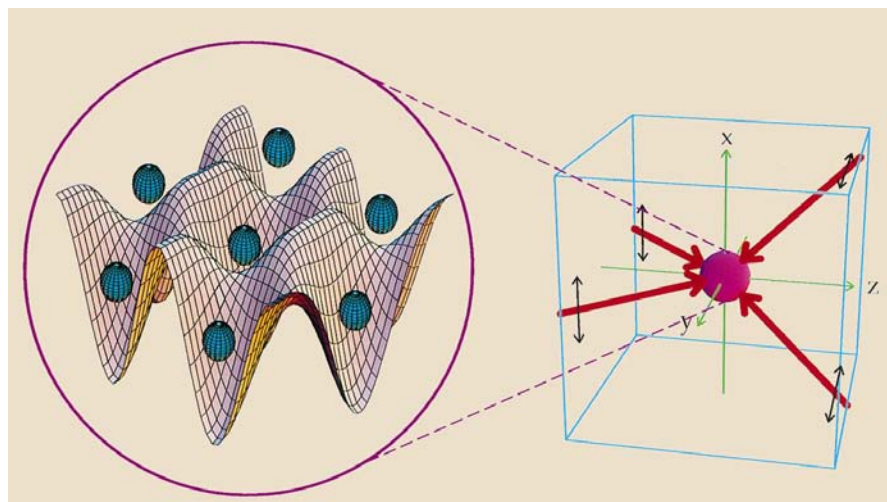


Figure 1 An optical lattice. Laser beams (red) intersect to form a standing-wave pattern of microscopic wells, which are about an optical wavelength apart and can hold individual atoms (left), and so manipulate them.

ence is degraded, reducing the intensity of the diffraction. The diffracted intensity is thus directly related to the atomic wavepacket width. Both groups observed that the intensity of the diffracted probe beam oscillated in time as the atom waves pulsed in the lattice.

A group at the University of Rochester in New York state³ observed similar oscillations in light scattered off sodium atoms, directly from the laser beams that make the optical lattice. When the atom wavepackets contract, they experience brighter regions of the lattice and so scatter more light; and vice versa. Common to all experiments, the scattered light undulations occurred at roughly twice the rate of rocking motion — which is the tell-tale sign of breathing motion.

In a bowl of water suddenly squeezed, the breathing motion is damped strongly by dissipation and dispersion of the waves. Similar effects wash out atom-wavepacket breathing in the optical lattices, limiting the number of observable oscillations to just a few. Although this decay does give valuable information about dissipative interactions between the atoms and the lattice, it can also mask interesting quantum effects of the motion which do not occur in water waves, such as quantum 'revivals' of the oscillation due to the anharmonic (non-parabolic) shape of the lattice wells. Nevertheless, the NIST group has very recently observed unambiguous quantum revivals in the rocking motion of the wavepackets⁶.

Optical lattices may someday find applications in lithography, where atoms could be released from the lattice sites and deposited onto a substrate to form useful miniature patterns. There remain many technical hurdles to cross before this is feasible, but because smaller is better in the lithography business, it is still worth investigating how small wavepackets can be shaped in an optical lattice. Heisenberg's uncertainty limit —

a trade-off between position and momentum uncertainty — places a natural quantum boundary on the size of a stationary atom wavepacket placed in the bottom of a well, a size that depends on the depth of the well. In typical optical lattices, this lower limit is around 10 to 100 nanometres.

But by shaping the atom wavepackets using the above techniques, the atoms can in principle be transiently squeezed down to much smaller sizes. Atoms in optical lattices

have not yet been squeezed below the quantum boundary, although related work in ion traps has allowed the squeezing of a single atom's wavepacket to about 1 nanometre — almost a factor of ten below the quantum boundary⁷.

Regardless of the prospects for atomic lithography, physicists are discovering many tools for harnessing atoms in this exotic form of matter. Several groups are now investigating optical lattices that are nearly free of dissipation and may more fully expose the quantum features of atomic motion, and allow squeezing well beyond the quantum boundary. As these recent atom-shaping experiments illustrate, optical lattices are becoming attractive playgrounds for quantum mechanics. □

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Protein folding

Folding with a two-stroke motor

George Lorimer

One of the hot problems in modern biology is how the molecular chaperone GroEL, along with its cohort GroES, facilitates the folding of other proteins. It is a wonderfully complex and highly dynamic process, the details of which are only beginning to emerge. Two papers by Xu *et al.*¹ and Rye *et al.*² from Yale University (pages 741 and 792 of this issue) mark considerable progress in our understanding, and again demonstrate the power of crystallography¹ coupled to clever mutagenesis². They also remind us that we still have lots to do before the mechanism of this remarkable two-stroke folding machine can be considered to be 'defined'.

The interaction between GroEL and GroES is necessary for certain proteins to fold under otherwise non-permissive conditions³. The subunits of both proteins are heterologously assembled into heptameric rings^{4,5}. In the case of GroEL, two rings stack back-to-back (isologously) to form the native 14-mer, enclosing two large, non-contiguous central cavities. Each monomer unit consists of three domains. The larger 'equatorial' and 'apical' domains are linked

through a smaller 'intermediate' domain.

GroES resembles an upturned saucer⁵, with seven unstructured and mobile loops⁶ extending from the rim. In the presence of nucleotide⁷, GroEL and GroES interact through the mobile loops to form both an asymmetrical GroES₇–GroEL₁₄ complex⁸, the structure of which is now reported by Xu *et al.*, and a symmetrical GroES₇–GroEL₁₄–GroES₇ complex^{9,10}.

Three-dimensional reconstructions of electron-microscopic images at 30 Å revealed that, accompanying the binding of ATP and GroES, there is a massive upward movement of the GroEL apical domains¹¹. Xu *et al.* now show that this is due to a twisting, en-bloc movement of the apical domains around a 'hinge' at the junction of the intermediate and apical domains. This movement creates an enlarged cavity — known as Anfinsen's cage (Fig. 1) — in which the unfolded substrate protein, at least transiently, resides^{12–14}. But the system is dynamic, and when the GroES cap¹⁴ dissociates from the proximal *cis* GroEL ring with each cycle (as occurs when ATP binds to the distal *trans* GroEL ring²), the substrate pro-

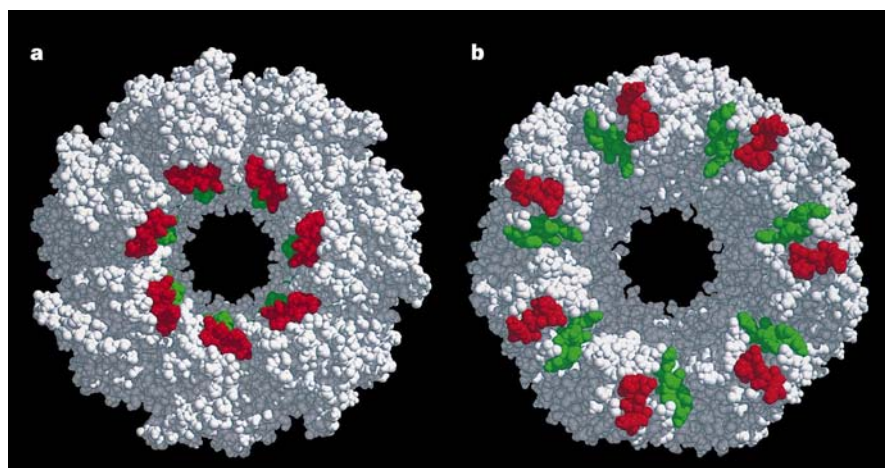


Figure 1 The crystal structure of the asymmetrical GroES7–GroEL14 complex, solved by Xu *et al.*¹, provides new insights into how GroEL and GroES mediate the folding of other proteins. GroES has been removed for clarity, and the complex is viewed from *trans* (a) and *cis* (b) sides. Helices H and I (coloured red and green, respectively) mark the binding site for the substrate protein. On the *cis* side they are further apart from one another than on the *trans* side, and their orientation also differs by about 90°. This movement creates a greatly expanded cavity on the *cis* side, in which the substrate protein is encapsulated. (Courtesy of Z. Xu, Yale University.)

tein is ejected from the cavity^{12–16}. This occurs irrespective of whether the protein has folded. So, the door to Anfinsen's cage, it seems, opens every 20 seconds or so to allow the occupants to escape — whether they have served their time or not.

The binding of non-native proteins to GroEL is largely nonspecific¹⁷ and hydrophobic in nature¹⁸. The binding site has been mapped to the apical domain^{8,19} — specifically, to a ring of hydrophobic residues lining the walls of the cavity. Curiously, some of the residues required for GroES binding map to the same region¹⁹. A more detailed view of this region has emerged from the crystal structure (1.7 Å) of a monomeric mini-chaperone²⁰ that comprises only the apical domain. It is almost identical to the apical domain of intact GroEL. By serendipity, an unstructured peptide was found to occupy part of the peptide-binding site, within the most flexible and disordered region of GroEL^{4,20}. So GroEL is equipped with a flexible, largely hydrophobic surface that can promiscuously accommodate a variety of unfolded proteins. One size fits (almost) all!

It has been suggested^{13,14,21,22} that GroEL unfolds misfolded proteins which, on release, are given another opportunity to reach the native state. Indeed, increased amide proton-exchange (indicative of unfolding) is catalysed by GroEL²³. However, it happens much too slowly to be part of an ATP-dependent, chaperonin folding cycle, which needs about 20 s for completion. Yet, somehow, this cycle of binding and release — synchronized to ATP hydrolysis by the GroEL rings¹⁴ — facilitates folding. The question is how. The structure of the asymmetrical complex solved by Xu *et al.*¹ provides some of the answers.

Comparing the structure of the asymmetrical complex with that of GroEL alone, two massive, rigid domain movements can be seen. In the complex, the apical domain has moved upwards away from the equatorial plate by some 60°, as well as twisting through 90°. This movement, which Rye *et al.*² show to be triggered by the binding of ATP to the equatorial domain, delivers the 'power stroke' in the folding cycle. In the closed conformation, the protein binding sites of adjacent subunits are about 25 Å apart (Fig. 1). But in the complex this distance has increased to about 33 Å (Z. Xu, personal communication) — so the seven bind-

ing sites move apart. Presumably, a protein bound to these sites will be subject to mechanical stress during this movement, and it will unfold to some unspecified degree.

The substrate protein is eventually displaced from its binding sites in a vectorial manner, so that it becomes encapsulated in the now-expanded cavity beneath the GroES cap. How this happens is unclear, but a simple two-step mechanism can be envisaged. The surface of the GroEL protein binding site comprises two overlapping regions, A and B (Fig. 2). On formation of a GroEL–GroES complex, region A becomes the interface with GroES — including the mobile loop — whereas region B becomes part of a new GroEL subunit–subunit interface. If the protein is displaced from site A (by GroES) before it is displaced from site B, encapsulation will be ensured. Speculation aside, Xu *et al.* find that the cavity into which the substrate protein is discharged is lined with polar residues. Here, the protein (which is now in a more unfolded, or higher-free-energy state) is free to fold (or misfold) on its own. That is, it seeks free-energy minima²² anew and, as Anfinsen told us, the primary sequence specifies the fold.

But this is a folding machine with a built-in clock, which is set to free the substrate protein — folded or not — every 20 s or so. How does this work? The second rigid-body domain movement that occurs when the asymmetrical complex forms, is a 25° shift of the intermediate domain towards the equatorial domain. This brings a new ligand, residue D398, into the coordination sphere of the Mg(II) that is associated with the phosphoryl groups of ATP^{1,24}. This residue

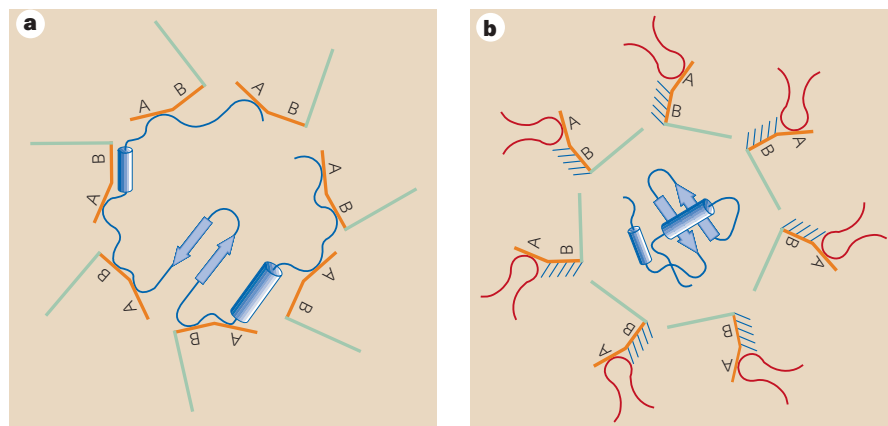


Figure 2 Rye *et al.*² have used mutagenesis to explore the mechanism of protein folding by GroES/GroEL. a, A stylized version of the apical domain of a GroEL ring, viewed down the seven-fold axis. A molecule of substrate protein (blue) is bound at several of the hydrophobic (orange) sites lining the cavity. Some secondary structure may remain in the substrate. Each site contains two overlapping subsites A and B, distinguished by their position in the asymmetrical complex. b, On binding ATP, the apical domains of each subunit twist by 90°. The substrate protein is displaced into the lumen of the cavity, the surface of which (green) has assumed a polar nature. Subsite A of the protein binding site now engages the mobile loop (red) from the underside of GroES, whereas subsite B forms a new subunit–subunit interface (hatched). The encapsulated protein is free to fold (or misfold) for about 20 s when ATP binding to the distal ring (not shown) triggers its discharge. Rebinding of non-native protein initiates a new cycle.

is probably involved in ATP hydrolysis because, as Rye *et al.* show, a mutant (D398A) has only 2 per cent of wild-type ATPase activity. Furthermore, when the equatorial and apical domains are reversibly crosslinked²⁵ so that D398 cannot move into the catalytic site, GroEL can bind ATP, but neither ATP hydrolysis nor GroES binding occur.

So, four events — ATP binding, domain movement, GroES binding/encapsulation of substrate protein and ATP hydrolysis — are linked. Because domain movement and GroES binding occur in the D398A mutant, it must be ATP binding in the *cis* ring, and not hydrolysis, that triggers the power stroke². The movement of D398 into the active site (which is effectively secured by the binding of GroES), sequesters the nucleotide and precludes exchange of ATP (or ADP) with free ligand. So, only in the presence of GroES is ATP committed to hydrolysis¹⁴ and, in this way, GroES ensures that all seven ATPs are hydrolysed as a unit. In effect, the reaction becomes 'quantized' in the presence of GroES¹⁴.

Operation of the timer requires ATP hydrolysis in the *cis* ring, which primes the ring to release its ligands. The actual release is triggered by events in the distal *trans* ring¹⁴. Rye *et al.* confirmed this using the single-ring mutant SR1, which forms a complex with GroES and performs only one round of ATP hydrolysis. It is thus primed to release its ligands but, lacking a distal ring, it does not receive the signal to do so.

What is the signal from the distal ring and how is it transmitted across the equatorial plate? Rye and colleagues' mutant analysis again points to ATP binding (but not hydrolysis, as previously suggested¹⁴) in the distal ring as the precursor to discharge of the ligands in the *cis* ring. Xu *et al.*¹ also report a subtle change in the orientation of the equatorial plate in the asymmetrical complex. In the *cis* ring, the subunits are tilted inwards by 4° so that the centre of the plate is some 8 Å lower than the rim. In the *trans* ring, the subunits are tilted outwards by 2° so that the interface between the rings is largely maintained. Such reciprocal shifts in the orientation of the equatorial plate — perhaps part of the power stroke — can readily be envisaged.

Those who appreciate symmetry in biology^{9,10} will note that if, besides ATP, substrate protein¹³ and GroES are added to this distal *trans* ring of the asymmetrical complex, to form a symmetrical complex, maximum efficiency can be achieved by combining the 'power stroke' in one ring with the signal that tells the other ring when it's time to release its ligands^{13,26}. The Yale 'football' team will surely want to solve the structure of the symmetrical complex too! □

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Demography

Death and the demon drink in Russia

If graphic illustration were needed of the poor state of public health in Russia, this figure depicting life expectancies between 1984 and 1994 provides it. It also shows striking fluctuations over the period concerned.

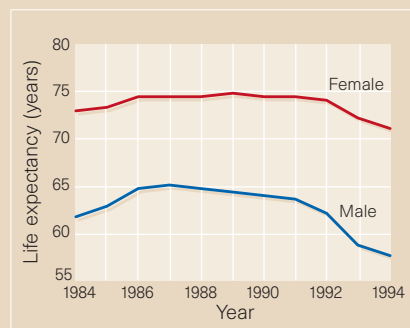
After an increase up to the mid-late 1980s, life expectancy for both men and women has decreased alarmingly; in 1994, for instance, male life expectancy at birth was 57.6 years, a decline of 7.3 years from 1987.

But why has there been such a dramatic variation? Could it be just an artefact, for instance stemming from erroneous estimates of the population size?

A group of demographers, based in London and in Moscow, published their analysis of the issue earlier this month (D. A. Leon *et al.* *Lancet* **350**, 383–388; 1997). They believe that the answer lies in the demon drink — in first a decrease and then an increase in the consumption of alcohol.

The data they used came from a mortality series for Russia compiled by the Institut National d'Études Démographiques in Paris and the Centre for Demography and Human Ecology in Moscow, broken down by cause of death. Most strikingly, mortality due to cancers was almost stable over the ten-year period, and because of this Leon *et al.* argue that the statistics reflect real changes in life expectancy.

Factors that could be involved in the decline since the mid-late 1980s are the increasing prevalence of tuberculosis, changes in diet and poorer provision of health care. But the breakdown shows a massive increase from mortality due to alcohol-related diseases and accidents or violence, which often stem from alcohol



Life expectancy from birth in Russia, by sex and year, for the period 1984–94. Redrawn from data in Table 2 of the paper by Leon *et al.*

abuse, especially among 40–50-year olds.

Moreover, the authors correlate the rise then decline in life expectancy with patterns of alcohol consumption in Russia. Consumption started to fall in 1984; it fell even more quickly after the advent of a campaign the next year, which included state restrictions on the availability of alcohol. By the end of the 1980s, however, home brewing was on the rise, and in the next few years there was a relaxation of state restrictions and a relative fall in the price of alcohol.

Untangling cause and effect from demographic or epidemiological data is fraught with uncertainty. But at the least Leon and colleagues provide a persuasive case to answer. It is one that underlines the point that, in many parts of the world, improvements in health are less likely to be won by modern medicine than by changing environmental conditions or social habits — in this instance persuading drinkers to lay off the booze, difficult as that may be.

Tim Lincoln

Immunology

Inside the gearbox of the dendritic cell

Colin Watts

Dendritic cells are a group of 'professional' antigen-presenting cells that seem to hold the key to the induction of T-cell responses. For this reason, there is a great deal of interest in their fundamental properties and how they might be used for immunotherapy — for example, to stimulate immune responses against tumours or infectious agents. Two papers by Cella *et al.*¹ and Pierre *et al.*² (pages 782 and 787 of this issue), reveal how environmental factors can trigger dendritic cells to reorganize and redirect their antigen-processing apparatus, to maximize their antigen-presenting and, hence, T-cell triggering capacity.

T lymphocytes are activated upon recognition of cell-surface major histocompatibility complex (MHC) class I and class II molecules carrying specific, processed, antigenic peptides. Although MHC molecules are expressed by many different cell types, only professional antigen-presenting cells and, in particular, dendritic cells, can trigger naive T cells³. Once over this hurdle, however, activated T cells can engage other cells expressing the same specific peptide–MHC class I or class II complexes.

The new studies^{1,2} are not the first to

report that dendritic cells modulate their antigen-presenting capacity upon maturation. Earlier work^{4–7} showed that, when these rare cells were isolated from tissues such as skin or spleen, a terminally differentiated population was obtained. These cells expressed high levels of MHC molecules on the cell surface, but had low rates of new MHC biosynthesis. As a result, they stimulated T cells that could recognize the existing surface MHC–peptide complexes (generated either *in situ*, or soon after tissue disruption), but they were poor at capturing and presenting newly offered antigens.

Later studies showed that this switch from an antigen-capturing mode to an antigen-presenting mode — could be induced in cultured dendritic cells by cytokines such as tumour-necrosis factor- α , and bacterial products such as lipopolysaccharide^{8,9}. These agents also induced the dendritic cells to migrate, *in vivo*, from non-lymphoid tissues to the T-cell-rich areas of lymphoid organs^{10,11}. So, the life history of the dendritic cell is played out in two parts. First it acts as a 'sentinel', sampling and processing the local milieu. Later, following migration, it

becomes a reporter of its earlier environment, which is expressed in peptide form on class II (and probably class I) MHC molecules. At this mature stage there are also elevated levels of adhesion molecules and 'co-stimulators' of T-cell triggering.

Although there are clear echoes of the earlier studies, Cella *et al.*¹ and Pierre *et al.*² have now reconstituted the distinct stages of the dendritic-cell life history under controlled conditions *in vitro*. They also provide new insights into the organization of class II MHC maturation, expression and stability at each of these stages. Both studies exploit methods for generating immature dendritic cells *in vitro*, either from blood monocytes using granulocyte–macrophage colony-stimulating factor (GM-CSF) and interleukin-4 (ref. 8), or from proliferating bone-marrow precursors¹² using GM-CSF.

For successful expression of antigenic peptide–MHC class II complexes on the cell surface, the endocytosed antigen needs to be efficiently mixed with newly synthesized class II MHC molecules, in specialized compartments of the endosome/lysosome system (reviewed in ref. 13). Proteases and specific regulators of peptide loading are also required. The assembled complexes must then be transported to the cell surface, where they should ideally be long-lived to maximize the opportunity for interaction with T cells. The work of Cella, Pierre and their colleagues now shows that dendritic cells can modulate these parameters to control antigen presentation.

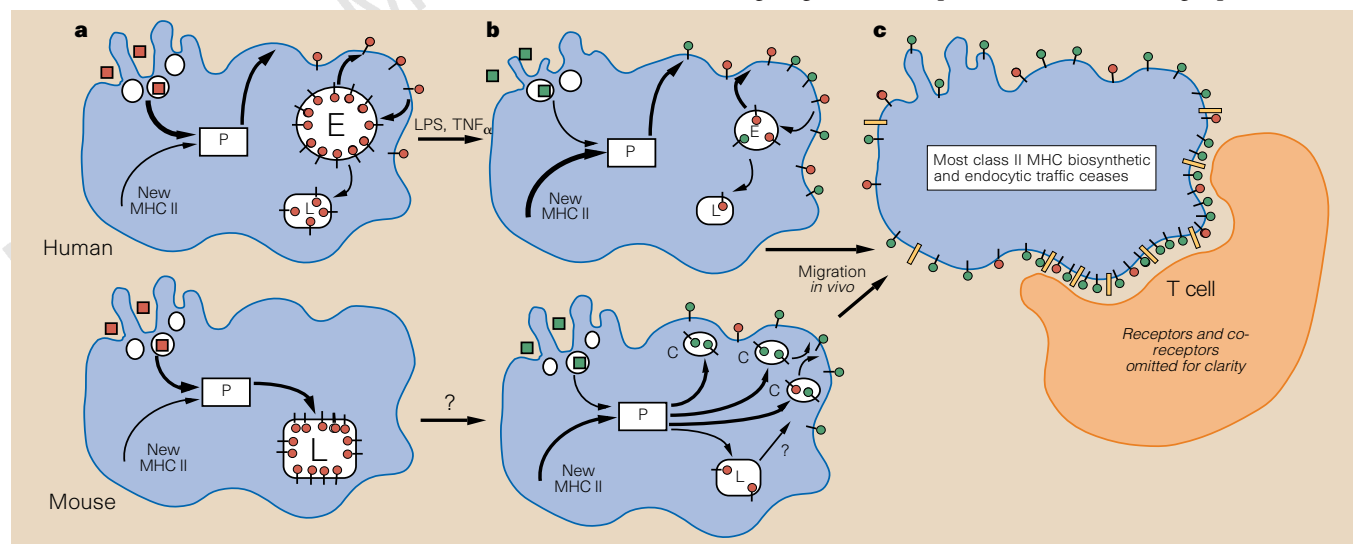


Figure 1 Dendritic cells hold the key to T-cell responses — they present antigenic peptides complexed with class II major histocompatibility complex (MHC) molecules to T cells. Cella *et al.*¹ and Pierre *et al.*² have used human and mouse dendritic cells, respectively, to work out how this is regulated as dendritic cells mature. In response to environmental factors (especially those that are normally associated with pathogenic invasions), dendritic cells reorganize themselves, to maximize their ability to present antigen. In an 'idling' immature dendritic cell (a), endocytosis (red squares) and MHC biosynthesis occur, but the peptide–MHC class II complexes (red ball and stick) accumulate in lysosomes (mouse), or reach the cell surface but are then recycled (human). In response to bacterial products (such as lipopolysaccharide, LPS; cytokines such as tumour-necrosis factor- α , TNF- α ; or as-yet undefined factors), the dendritic cells change gear and start to mature (b). There is increased biosynthesis of class II MHC, which maximizes specific loading of antigen (green squares). Peptide loading may occur in diverse compartments, including CIIV^{2,14} and recycling endosomes¹. In the mature dendritic cell (c) the peptide–MHC class II complexes are expressed efficiently on the cell surface (green ball and stick), where they have a dramatically extended half-life because endocytosis is shut down. Increased levels of adhesion and co-stimulatory molecules (yellow) complete the surface phenotype that is needed to trigger naive T cells. Antigen that is captured in the idling and/or maturing state can therefore be very efficiently presented to T cells in the maturing state. P, compartments of peptide loading; E, recycling endosome; L, lysosome; C, CIIV-like endosome.

A key finding in both studies is that, although immature dendritic cells are active in the biosynthesis of class II MHC and in peptide loading, the assembled peptide-MHC class II complexes are not stably expressed on the cell surface. Instead, they accumulate in lysosomes² or, in the human-monocyte-derived dendritic cells¹, the complexes reach the cell surface, but they are removed and become degraded relatively quickly. It is as though the machinery for antigen processing is running but, at this stage, the cell is set up in an 'idling' mode and has not found the right gear to drive the effective presentation of antigen to T cells. As shown in Fig. 1, the idling state differs between the two systems in terms of class II MHC routing and steady-state distribution.

Maturation — induced by microbial products or cytokines in the human cells, and by as-yet unidentified factors in the murine cultures used by Pierre *et al.*² — engages the correct gear to drive the expression of class II MHC molecules onto the cell surface. Maturation also transiently accelerates class I and class II MHC biosynthesis, particularly in the human cells. In the dendritic cells from murine bone marrow, the class II MHC is redistributed from lysosomes in the immature cells, to peripherally located endocytic vesicles. These resemble a specialized class II MHC-rich population (CIIV), previously found in B cells¹⁴, which are constitutively active antigen-presenting cells. Finally, fully mature cells in both systems express almost all of their class II MHC on the cell surface (Fig. 1).

How much of the class II MHC in the CIIV-like endosomes is salvaged from the earlier lysosome pool, and how much arises from new biosynthesis? Although Pierre *et al.* do not directly address this, newly assembled class II MHC molecules are now clearly directed to the cell surface, instead of to lysosomes. Importantly, the sequential changes in the distribution of class II MHC molecules seen in the murine bone marrow cultures are also observed *in vivo* in murine Langerhans cells migrating out of, or maturing within, explanted skin².

Both groups find that antigen captured by immature cells in an early time window can be presented by the same cells at a later stage, providing their maturation programme begins soon after antigen capture. Most peptide-MHC class II interactions are irreversible, so 'memory' of earlier antigenic encounters depends on the half-life of cell-surface class II MHC (ref. 15). This is increased up to ten-fold on mature, compared with immature, dendritic cells^{1,2}. A long half-life is important *in vivo*, as the immature cell migrates from the periphery — where it has captured antigen — to the lymphoid organs, where its antigenic load is scanned by T cells. The increased half-life is due to reduced endocytosis of class II MHC

(ref. 1), although it is not known whether this occurs through down-regulation of macropinocytosis¹⁶ or clathrin-mediated endocytosis, or both.

Cella *et al.*¹ also find that antigen offered during the transient boost in class II MHC biosynthesis (which is triggered, in this case, by tumour-necrosis factor- α) is loaded on class II MHC to a higher 'specific activity' than before. This means that microbial products 'help' to eliminate the pathogen, by stimulating the preferential presentation of microbial antigens. They may do this directly, through lipopolysaccharide, or indirectly through cytokines such as tumour-necrosis factor- α .

We now need to understand the molecular basis of the changes in class II MHC trafficking which characterize the maturation of dendritic cells. For example, why do class II MHC molecules accumulate in lysosomes in immature murine dendritic cells? At what level is this controlled? What drives the constitutive membrane ruffling and endocytosis in immature dendritic cells¹⁶, and how is this shut off in mature cells? The search for gene products that are differentially expressed in mature versus immature dendritic cells is already under way. Some may be relevant to these changes.

Finally, these studies may be relevant to the use of dendritic cells for clinical immunotherapy. The hope is that dendritic cells — obtained *in vitro* from patients' blood — could be loaded with known or putative tumour antigens, and then used as immunogens to elicit anti-tumour cytotoxic T-cell responses. Studies in animal models have already shown the potential of this approach (reviewed in ref. 17). Related strategies, such as direct fusion of tumour cells with dendritic cells, have also been described (see the News and Views article by Hart and Colaco on pages 626–627 of last week's issue).

Tumour-antigen-feeding protocols designed to generate specific class I and class II MHC T-cell determinants on cultured dendritic cells could take advantage of both the transient boost in MHC biosynthesis which is seen at the outset of maturation, and the dramatically prolonged half-life of class II helper determinants on fully mature cells. □

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Daedalus

Magnetic autoeroticism

Last week Daedalus presented a magnetic nerve stimulator. It launched magnetic pulses into the spinal cord or other nerve pathways. Its pulses could be tailored in shape and frequency to induce firing currents only in specific nerves — say, those to a certain muscle. The user could thus control that muscle more precisely.

He is now applying the technology to muscles we cannot control at all. A magnetic heart pacemaker, for example, need not be implanted. It could be strapped on the chest, from where it could be adjusted at will. If the wearer wished to run for a bus, he could speed up his heart for the extra effort. Similarly, the widespread miseries of constipation could be cured by a magnetic belt, tuned to the key parasympathetic nerves in the spinal cord. The wearer could defecate on command, simply by pressing a button.

The most widespread miseries of them all, muses Daedalus, are probably sexual. A magnetic belt tuned to the appropriate spinal nerves could induce sexual arousal and even orgasm on command. It would transform the lives of those trapped in unhappy or fading relationships. But too much sex is probably as troublesome as too little. Thus, rape, murder, robbery and pop-music composition are all forms of sexual behaviour: they are overwhelmingly the work of young, sexually driven males. Such offenders are already being marked by special electronic tags. Daedalus proposes a 'neuromagnetic tag', again in spinal belt form, to control the wearer's sexuality. The obvious idea is to block it completely. But Daedalus favours the converse approach. His neuromagnetic tag will stimulate the nerves of orgasm as frequently and intensely as possible. This will exhaust the wearer's sexual drive. Without any other stimulation, he will find himself having repeated involuntary orgasms as fast as his physiology can manage it. A rapist or sex offender would be permanently drained, 'shorted out' and made harmless by such a tag. And if sex underlies the rest of the portfolio of male behaviour, then murder, robbery, pop-music composition and other male social nuisances would be curtailed too. Their exponents could safely be released into the community. At intervals they would show a certain sudden inwardness and self-absorption, but would otherwise be quiet, undisturbed citizens. The treatment could not even be condemned as a cruel and unusual punishment. It would be a pleasurable and unusual punishment.

David Jones

Obituary

John Zachary Young (1907-97)

Zoologist who paved the way for modern studies in neurobiology

John Young, who died on 4 July aged 90, was one of the most distinguished biologists of the century — he himself made several seminal discoveries, and he had a great influence on the work of many others either directly or through his writings.

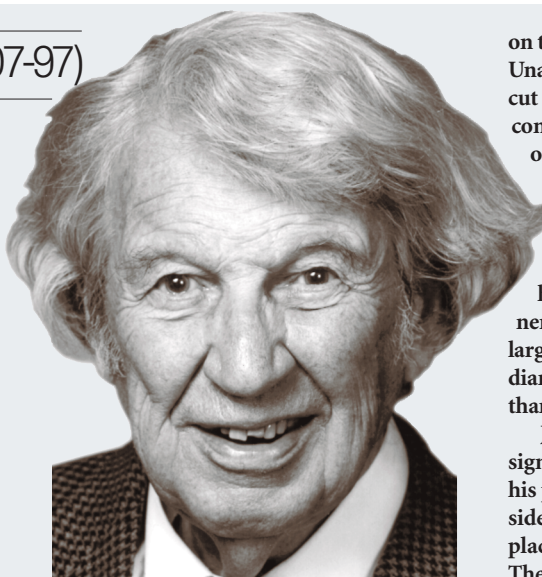
A zoologist by training and by inclination, JZ (pronounced the English way, 'Jay Zed') was passionately interested in how animals work, especially in the operation of their brains. But his restless curiosity and disdain for conventional academic boundaries led him to work in, and write about, areas that others might label physiology, neuroscience, experimental psychology or philosophy. He was eclectic but did not hesitate to go deep (as he used to put it) into whatever was his interest of the moment. Many are the collaborators who can vouch for his amazing grasp of detail in what they had hitherto thought of as their own subject area.

Born in Bristol, JZ was educated at Marlborough College and Oxford University, where he became a fellow of Magdalen College before being appointed to the chair of anatomy at University College London in 1945.

The breadth of his achievements can be shown by considering his work — first as a cephalopod biologist, then as a teacher and, finally, as a neuroscientist. For some zoologists, JZ was an outstanding authority on cephalopods (the octopuses, squids, cuttlefishes and nautilus). His first scientific paper, published in 1929, was about an organ that he had discovered on the stellate ganglion of the octopus. For nearly 70 years he continued to write papers and books about these animals, and many of them have long been classics.

JZ also wrote many papers about his experiments on octopus learning, in which he tried to establish the basis of memory. With his collaborators, he showed that octopuses have distinct stores in the brain for visual and touch memories, and that each of these has a complex arrangement of accessory lobes that help to establish memories and gain access to them. But the octopus brain proved to be far more complicated than he originally envisaged, and this aspect of his work languished in recent years.

For a far wider group of students and zoologists, JZ was a teacher — a textbook



writer *par excellence*. His *Life of Vertebrates* (1950), *Life of Mammals* (1957) and modestly titled *Introduction to the Study of Man* (1971) each became, and remain, standard texts, widely translated. And it is not difficult to see why. They were well illustrated, authoritative, comprehensive and, above all, beautifully written. The ability to express himself simply was one of JZ's greatest gifts, and it made him a brilliant lecturer as well as writer. He brought to his lectures an excitement and an infectious enthusiasm that came from the heart. There was absolutely no pretence in him: he found animals and brains so fascinating (one of his favourite epithets) that he could not but convey that fascination to his listeners.

He was also a teacher in the broader sense, eager to convey to a wide audience the very essence of science (witness the title of his 1950 Reith Lectures: *Doubt and Certainty in Science*), and bold enough to speculate about the possible wider implications of an experimental result. This boldness runs through his later exploratory works, which differ from the writings of many superannuated scientists in that they make no concessions to mysticism and are remarkable for their humility. He recognized the absurdly wide canvas on which he was working, but felt that it was his duty to attempt it. JZ was convinced that understanding the human condition must involve studying the organ of study itself — the brain.

Finally, for neuroscientists of all kinds, JZ was the person who, in the 1930s, discovered the giant nerve fibres of squids, paving the way for the experiments that established how nerves transmit information. He made that discovery because, purely through intellectual curiosity, he looked for the squid equivalent of the organ that he had found

on the stellate ganglion of the octopus. Unable to see it under the microscope, he cut sections of the squid stellate ganglion to confirm its absence, and observed a series of enormous, tubular structures that he at first took to be blood vessels.

However, further observations and simple but crucial electrical stimulation experiments convinced him that each 'tube' was, in fact, a single nerve fibre — a so-called 'giant' fibre. The largest such fibres were nearly 1 mm in diameter and over a hundred times fatter than a mammalian nerve.

JZ immediately recognized the significance of his discovery. He showed his preparation to physiologists on both sides of the Atlantic, who succeeded in placing electrodes inside the giant fibre. They established that there was a small potential difference between the outside and the inside, and that this reversed when a signal passed along the axon. In 1963, Alan Hodgkin and Andrew Huxley were deservedly awarded a Nobel prize for their elegant experiments on the squid giant fibre, but there are many who thought that the award might have been extended to include JZ.

What kind of person was it who could achieve all of this? Complex, certainly — a curious mixture of self-confidence and humility, impatience and perseverance. With his indomitable drive to achieve, he could be irascible and inconsiderate, and he was often genuinely puzzled by the behaviour of those living life at a slower pace. Once, at the Naples Zoological Station, he rebuffed a visitor for trying to speak to him as he was walking down a corridor, and later complained, "Can't people see you're going somewhere?". Yet, having gained his attention, you would encounter immense courtesy and charm.

He was perhaps at his best in the pub after a hard day's work, talking enthusiastically about his results and yours, sharing his passion for the science that was his life. These attitudes stemmed from his socialist principles and deep humanism. The patrician style went hand in hand with the common touch: he loved the warm-hearted people of Naples, and of the many honours that came his way he especially cherished the honorary citizenship that this city bestowed on him in 1991.

All of us in his 'scientific family', as he called us at his ninetieth birthday party in April, mourn his death. But we remember his life and cherish the privilege of having known him.

John Messenger

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Hyperactive antifreeze protein from beetles

We have purified a thermal hysteresis (antifreeze) protein, with up to 100 times the specific activity of fish antifreeze proteins, from the common yellow mealworm beetle, *Tenebrio molitor*. It is a threonine- and cysteine-rich protein, of relative molecular mass 8,400, composed largely of 12-amino-acid repeats. We estimate that a concentration of roughly 1 mg ml⁻¹ of this protein can account for the 5.5 °C of thermal hysteresis found in *Tenebrio* larvae (Fig. 1).

Although protein-mediated thermal hysteresis was noted in *Tenebrio* almost 30 years ago¹, numerous attempts to purify any thermal hysteresis proteins (THPs) failed to yield activities (quantified as the temperature difference between the freezing and melting points of a solution containing ice) sufficient to account for that of haemolymph¹⁻⁵. We have now isolated microgram quantities of THP from diluted larval *Tenebrio* haemolymph by gel exclusion chromatography followed by reversed-phase high-performance liquid chromatography (HPLC) on an analytical C18 column. Neighbouring HPLC fractions corresponded to discrete proteins (THP26 and THP27). The thermal hysteresis activity of THP26 at 55 µg ml⁻¹ was 1.6 °C and of THP27 at 21 µg ml⁻¹ was 1.1 °C, close to the maximum values obtained with 10–30 mg ml⁻¹ of fish antifreeze proteins (AFPs)⁶.

Because the amino termini of THP26 and THP27 were blocked, we sequenced an internal fragment from THP26 that was released by endoproteinase Lys-C digestion (Fig. 2). We used degenerate and vector primers in the polymerase chain reaction and screened a *Tenebrio* complementary DNA library with the product. We obtained the complete coding sequences of four THP variants (YL1–4), whose conceptual translations match the sequenced peptide fragment at up to 18 of the 20 residues (Fig. 2). The first 28 amino acids represent a secretory signal peptide. The N-terminal amino acid of three of the variants is predicted to be glutamine, consistent with N-terminal blockage in which the N-terminal glutamine is converted to pyroglutamate by cyclization. The cleavage site of the fourth variant is not clearly predicted.

The amino-acid compositions of the purified proteins and the deduced mature sequences are very similar. The THPs are particularly rich in cysteine (18–19%), threonine (21–26%) and other amino acids with short side chains (glycine, alanine, serine), and are deficient in several hydrophobic amino acids, notably leucine and isoleucine.

The overall hydrophilicity is roughly 55%⁷, much higher than that of fish AFPs⁸.

The primary structure of the proteins (Fig. 2) is very unusual and unlike any previously known sequence. The first 21 amino acids contain six cysteine residues spaced at irregular intervals (Cx₅Cx₂Cx₃Cx₂Cx₂C), and this sequence overlaps with the first of a series of 12-amino-acid repeats, in which cysteine is repeated at six-residue intervals, that continue until the end of the protein. The consensus of these repeats is CTxSxxCxxAxT. The THP variants differ in the number of these repeats. Additional 12-amino-acid units appear as insertions in the longer variants, indicating that each may form a functional domain and that the exact order of the repeats might not be crucial for function.

We expressed the shortest of the four cDNAs with a conventional signal cleavage site (YL1) in *Escherichia coli* (Fig. 2). We detected thermal hysteresis in the supernatant of the cell lysate, indicating that some of the protein was able to fold into an active state. Partially purified recombinant

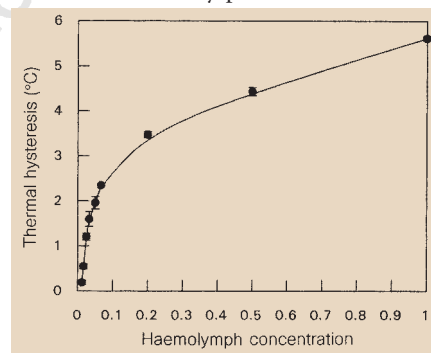


Figure 1 Thermal hysteresis activity of *Tenebrio* haemolymph as a function of dilution. The standard deviation of each sample ($n=3$) is shown.

Figure 2 Predicted amino-acid sequences of four THP variants obtained from a fat-body cDNA library. The predicted N-terminal residues (+1) are denoted by an arrow with the signal peptides shown in italics. The repetitive 12-amino-acid domains are numbered consecutively. Conserved residues found in almost all repeats are indicated in bold. The amino-acid sequence from a purified THP fragment (THP26) is shown for comparison. The first Lys residue is assumed as the protein was cleaved with endo-Lys-C. Undetermined positions were assumed to be Cys. The two amino-acid regions used to design the nested degenerate PCR primers are underlined. Databank accession numbers for the THP sequences YL1–4 are AF010329, AF010330, AF010331 and AF010332, respectively.

THP showed thermal hysteresis of 5.3 °C and its properties were indistinguishable from the THP in *Tenebrio* haemolymph. Ice crystals that formed in the presence of haemolymph and recombinant protein were identical (Fig. 3 a,b). This and other THP isoforms could account for all thermal hysteresis activity in the insect, because THP *in vitro* does not need other factors to attain values of more than 5 °C.

Like fish AFPs, *Tenebrio* THP seems to act by an adsorption–inhibition mechanism, as ice crystals did not grow until the non-equilibrium freezing point was exceeded. Further, the relationship between thermal hysteresis and THP concentration is hyperbolic (Fig. 1) and qualitatively indistinguishable from that of fish AFPs⁹. However, ice crystals formed in the presence of THPs

YL1	M A F K T C G F S K K W L V	-15
YL2	<i>M</i> T A N F S K S T G C G F G S H K A G W L 84	-15
YL3	<i>M</i> T A N F S K S T G C G F G S H K A G W L 84	-15
YL4	<i>M</i> T S N F S K S T G C G F G S H K R 7 W L 95	-15
YL4	C T N S K T G C P G H R 9	120
YL1	I A V I V M C L C T E C Y C	-1
YL2	I A V I V M C L C T E C Y C	-1
YL3	I A V I V M C L C T E C Y C	-1
YL4	I A V I V M C L C N E Y N C	-1
	↓	
YL1	Q C T G G A D C T S C T G A	14
YL2	H C T G G A D C T S C T D A	14
YL3	Q C T G G A D C T S C T A A	14
YL4	Q C T G A A D C T S C T A A	14
YL1	C T G C G N C P N A V T	R1 26
YL2	C T G C G N C P N A H T	R1 26
YL3	C T G C G S C P N A H T	R1 26
YL4	C T G C G N C P N A I T	R1 26
THP26	C T N S Q H C V K A N T	4
YL1	C T D S K N C V K A A T	R2 38
YL2	C T D S K N C V K A A T	R2 38
YL3	C T D S K N C V R A E T	R2 38
YL4	C T G S K N C V R A T T	R2 38
YL1	- - - - - - - - - -	
YL2	- - - - - - - - - -	
YL3	C T D S E N C V K A H T	R3 50
YL4	- - - - - - - - - -	
THP26	C T G S T D C N T A V T	16
YL1	C T G S T D C N T A Q T	R3 50
YL2	C T G S T K C N T A R T	R3 50
YL3	C T G S R N C N T A M T	R4 62
YL4	C T G S T N C N R A T T	R3 50
THP26	C T N S K D C F E A N T	21
YL1	C T N S K D C F E A K T	R4 62
YL2	C T N S K D C F E A K T	R4 62
YL3	C T N S K D C F E A K T	R5 74
YL4	C T N S K G C L E A T T	R4 62
YL1	- - - - - - - - - -	
YL2	- - - - - - - - - -	
YL3	- - - - - - - - - -	
YL4	C T G S T H C H R A T T	R5 74
YL1	- - - - - - - - - -	
YL2	- - - - - - - - - -	
YL3	- - - - - - - - - -	
YL4	C T N S K D C F E A T T	R6 86
YL1	- - - - - - - - - -	
YL2	- - - - - - - - - -	
YL3	- - - - - - - - - -	
YL4	C T G S S N C Y T A T T	R7 98
YL1	C T D S T N C Y K A T A	R5 74
YL2	C T D S T N C Y K A T A	R5 74
YL3	C T D S T N C Y K A T A	R6 86
YL4	C T N S T N C Y K A T A	R8 110

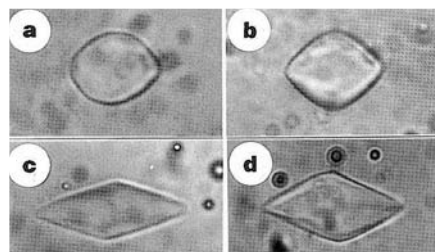


Figure 3 Photomicrographs of ice crystals grown in the presence of THP or AFP. The α -axis is horizontal in the plane of the page in all cases. **a**, Dilute haemolymph. **b**, Recombinant THP. **c**, Type-I fish AFP. **d**, Type-III fish AFP.

were unusual in that their surfaces were curved, whereas crystals generated by fish AFP types I and III are hexagonal bipyramids with flat, well-defined facets (Fig. 3 c,d), reflecting, in the case of type-I AFP, affinity for a specific pyramidal plane¹⁰.

The maximum observed thermal hysteresis value (5.5 °C) is four times that obtained with fish AFPs⁶. At low concentrations the activity is up to 100 times that of fish AFPs, consistent with our observations that THP is a minor constituent of haemolymph. Contamination with as little as 0.1–1% THP could produce activities comparable to those obtained with fish AFPs, suggesting that past THP preparations^{2–5} may have been impure.

A possible explanation for the hyperactivity of THP is that the protein has multiple ice-binding sites and that these sites either recognize several different features of ice, or a repeat expressed in several directions on the ice lattice. These properties could increase the frequency of THP binding to ice and might account for the curved ice crystal surfaces. The fourfold increase in maximum thermal hysteresis values might result from closer spacing of THP on the ice surface¹¹. The exceptional activity of *Tenebrio* THP makes it an attractive reagent for applications requiring freeze resistance or the control of ice growth and morphology.

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Role of CED-4 in the activation of CED-3

Genetic analyses of the nematode *Caenorhabditis elegans* have identified three core components of the cell-death apparatus¹. CED-3 and CED-4 promote, whereas CED-9 inhibits cell death. Recent studies indicate that CED-4 might interact independently with CED-3 and CED-9, forming the crux of a multicomponent death complex². But except for its role as an adaptor molecule, little is known about CED-4 function. A clue came with the observation that mutation of the phosphate-binding loop (P-loop) of CED-4 disrupts its ability to induce chromatin condensation in yeast³. Further, a P-loop mutant of CED-4 (CED-4^{K165R}) fails to process CED-3 *in vivo*, both in insect⁴ and mammalian cells (unpublished). We now confirm that CED-4 induces CED-3 activation and subsequent apoptosis, and that the process requires binding of ATP.

To test whether CED-4 could bind ATP, we used ATP analogues that label the nucleotide-binding sites on proteins. One such analogue is 5'-fluorosulphonylbenzoyl-adenosine (FSBA)^{5,6}. The natural nucleotide ATP (with Mg²⁺), effectively inhibited FSBA incorporation whereas CTP (with Mg²⁺) did not (Fig. 1a), showing that FSBA binds specifically to CED-4. The photoaffinity ATP analogue 8-azido-adenosine-5'-triphosphate [α -³²P] (8N₃-ATP) has also been used to identify ATP-binding proteins^{7,8}. As predicted, CED-4 bound 8N₃-ATP and this photoaffinity labelling was attenuated with ATP and MgCl₂ (Fig. 1b). Unlike wild-type CED-4, a form of CED-4 (residues 171–549) in which the P-loop motif was deleted (CED-4 Δ PL), failed to bind the azido analogue (Fig. 1c).

We next assessed the function of the CED-4 P-loop, and tested whether CED-4 activates CED-3 *in vitro*. We prepared

extracts from 293T cells transfected with CED-4 or the P-loop mutant CED-4^{K165R}. We saw proteolytic processing into active subunits when extracts containing CED-4 were incubated with *in vitro* translated ³⁵S-labelled CED-3, but not when incubated with the CED-3 active-site mutant, CED-3mt (Fig. 2a). Extracts generated from the vector or cells transfected with CED-4^{K165R} also failed to activate CED-3 *in vitro* (Fig. 2a). When CED-9 or CED-3mt were coexpressed with CED-4, CED-4 failed to proteolytically activate CED-3 (Fig. 2a).

To rule out the possibility that this phenomenon was due to a possible apoptotic nature of the CED-4-containing extract, we depleted CED-4 from the extract using Myc and histidine affinity tags. When we incubated CED-4-depleted extracts with CED-3, we found no CED-3 processing (Fig. 2a).

To confirm that CED-4 was responsible for CED-3 activation, we purified Myc/His affinity tagged CED-4 from the 293T extract by Ni²⁺ chromatography⁹. Purified CED-4, but not CED-4^{K165R}, stimulated CED-3 processing *in vitro*, and again the active-site mutant CED-3mt was not processed by CED-4 (Fig. 2b), implicating CED-4 as a catalyst for CED-3 self-processing.

In addition, a peptide inhibitor of caspases, DEVD aldehyde, abrogated CED-4-mediated auto-processing of CED-3. When the prodomain of CED-3 was truncated (CED-3(99–502)), CED-4 was unable to catalyse CED-3 processing (Fig. 2b). The prodomain of CED-3 interacts with CED-4 (ref. 10), and this CED-3 prodomain interaction with CED-4 seems to be required for CED-4-mediated activation of CED-3 processing. We also show that the ATP analogue FSBA blocks CED-4-mediated activation of CED-3 (Fig. 2c), confirming the functional importance of ATP binding in CED-4 activity as a catalyst for CED-3 self-processing.

We analysed the *C. briggsae* CED-4 sequence, and searched the non-redundant database, to find the highest similarity (after *C. elegans* CED-4) with the sequence

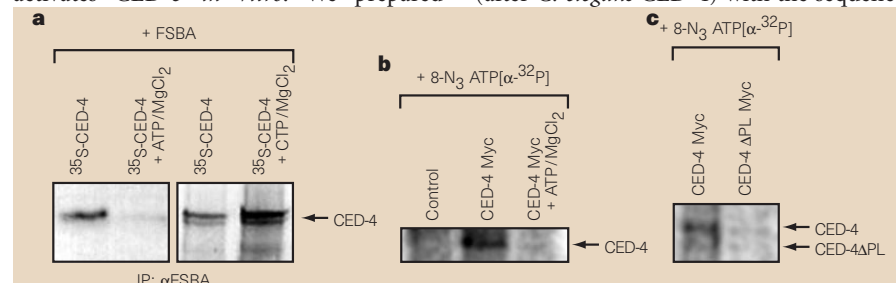


Figure 1 CED-4 binds ATP analogues. **a**, CED-4 binds FSBA, an inhibitor of P-loop-containing ATPases. ³⁵S-labelled CED-4 was incubated with 1 mM FSBA in DMSO in the presence or absence of excess ATP or CTP (10 mM) and MgCl₂ (10 mM), then immunoprecipitated with rabbit anti-FSBA (Boehringer), followed by SDS-PAGE. **b**, CED-4 binds an azido derivative of ATP. Lysates from 293T cells expressing Myc-CED-4 were incubated with 10 μ M (final concentration) 8N₃-ATP in the presence or absence of excess ATP (1 mM) and MgCl₂ (1 mM). For azido-affinity labelling, samples were irradiated⁷, and then immunoprecipitated with anti-Myc antibodies (Boehringer) followed by SDS-PAGE and phosphorimager analysis. Control represents lysates from vector-transfected cells. **c**, A P-loop deletion mutant of CED-4 fails to bind an azido derivative of ATP. Samples processed as in **b**. Expression of CED-4 and CED-4 Δ PL was confirmed by immunoblotting.

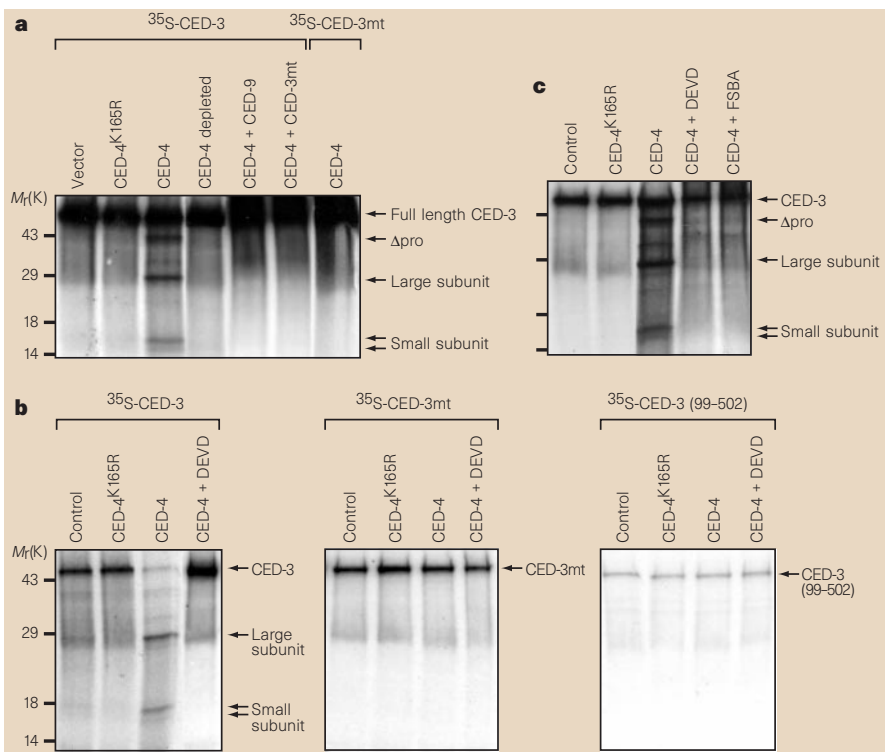


Figure 2 CED-4 facilitates CED-3 auto-activation *in vitro*. **a**, Cell extracts expressing CED-4 process *in vitro* translated, ^{35}S -labelled CED-3 but not CED-3mt. Myc/His-CED4 was depleted from an extract derived from CED-4-transfected 293T cells by incubation with Ni^{2+} beads and further depleted using anti-Myc antibodies and protein G. ^{35}S -labelled CED-3 was made by *in vitro* transcription/translation using the TNT rabbit reticulocyte lysate system (Promega; 30 min, 30 °C). Prolonged incubation of the *in vitro* translate triggered auto-processing of CED-3 (ref. 13) and so was avoided. Reactions used 5 μl ^{35}S -labelled CED-3 and 20 μl lysate containing the indicated proteins, incubated for 1 h at 30 °C. **b**, Purified CED-4 activates *in vitro* translated CED-3. CED-4 and CED-4 $^{\text{K165R}}$ were purified from transfected 293T cells⁸. Reactions used ~10–50 ng purified protein and *in vitro* translated ^{35}S -labelled CED-3, CED-3mt or CED-3(99–502) incubated for 1 h at 30 °C¹⁰. DEVD-aldehyde was at 200 nM. **c**, CED-4 activation of CED-3 is blocked by FSBA (1 mM). Methods as in **b**.

of the flax rust-resistance protein L6 (GenBank accession number 862905), a putative P-loop-containing ATPase¹¹. The alignment of the *C. briggsae* CED-4 and L6 sequences consisted of 322 amino-acids with 23% identical and 43% similar residues. When the predicted ATPase domain of L6 was used to search the non-redundant database with the PSI-BLAST (position-specific iterative BLAST) program, which enhances BLAST search with profile analysis¹², the similarity to *C. elegans* CED-4 was detected at a statistically significant level ($P < 10^{-5}$). Analysis of a multiple alignment of the CED-4 sequences with those of plant resistance-response gene products homologous to L6 (GenBank accession numbers 862905, 1842251, 1086263, 699495, 1931650 and 1513144) showed a consistent pattern of conservation which was particularly prominent in the P-loop, the putative Mg^{2+} -binding site, and two additional, downstream motifs (unpublished). CED-4 and plant-resistance gene products may contain a homologous and probably functionally analogous ATPase domain.

In summary, CED-4 is central to the regulation of the molecular framework of cell death. It has an intrinsic enzymatic activity

which facilitates CED-3 auto-activation and subsequent apoptosis. CED-3 activation by CED-4 requires the activity of the putative CED-4 ATPase domain. The presence of a conserved ATPase domain in CED-4 and the plant resistance-response proteins indicates that there may be similarities between the cell-death machinery and mechanisms in animals and plants.

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Vaccination onto bare skin

We report here that applying genetic vectors onto the skin in a relatively non-invasive manner can elicit an immune response against the protein encoded by the vector. This procedure requires no special skill or equipment and so may reduce medical costs and offer a unique method for vaccination.

To elicit a specific immune response, we anaesthetized mice and removed hair and cornified epithelium over a restricted area of abdominal skin using a depilatory (for example Nair)¹. We pipetted roughly 10^8 plaque-forming units (PFU) of adenovirus vector in a volume of 10–50 μl onto the pre-shaved and Nair-treated skin. We allowed the adenovirus vector to incubate with the naked skin until the animal recovered from anaesthesia, which took between 30 min and 1 h.

After application of AdCMV-hcea, an adenovirus vector encoding the human carcinoembryonic antigen (CEA) gene, onto the skin of mice (strain C57BL/6), we monitored the production of antibodies against the human CEA protein by assaying sera from tail-bleeds. The test sera reacted in western blots with purified human CEA protein, but not with bovine serum albumin (Fig. 1a). Of 24 vaccinated mice, 23 (96%) produced antibodies against the human CEA protein after a month, indicating that specific antibodies were produced against exogenous proteins encoded by adenovirus vectors as a result of the treatment. Antibodies against adenoviral proteins were also found in some of the treated animals.

To test whether this technique might be generally applicable, we applied AdCMV-hgmcsf, an adenovirus vector encoding the human granulocyte macrophage colony stimulating factor (hGM-CSF), in the same way. The test sera reacted with purified human GM-CSF protein (Fig. 1b) in 6 of 14 mice showing that 43% produced antibodies against the human GM-CSF protein after application of AdCMV-hgmcsf. Pre-immune sera collected before treatment, sera from untreated animals, and sera from animals treated with AdCMV-luc² all failed to react with the human CEA and GM-CSF proteins.

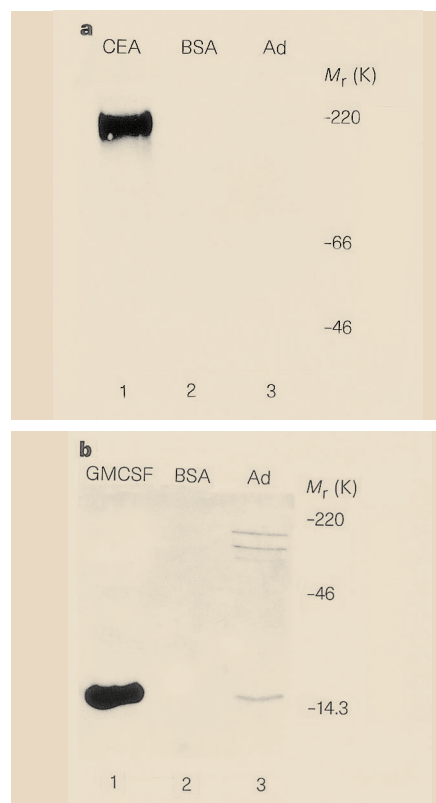


Figure 1 **a**, Detection of antibodies to the human CEA protein by western blot analysis. Serum from a vaccinated mouse taken one month after applying AdCMV-hcea to the skin was diluted 1:500 and reacted with purified human CEA protein (provided by T. Strong) and adenoviral proteins separated on a 5% SDS-polyacrylamide gel. The products were transferred to membranes as in ref. 3. Lane 1, human CEA (0.5 µg); lane 2, BSA (0.5 µg); lane 3, adenovirus (10⁷ PFU). **b**, Detection of antibodies against the human GM-CSF protein. Purified human GM-CSF protein (CalBiochem), separated on a 15% SDS-polyacrylamide gel, was transferred to membranes and allowed to react with diluted serum. Lane 1, human GM-CSF (0.25 µg); lane 2, BSA (0.25 µg); lane 3, adenovirus (10⁷ PFU).

To our knowledge, this is the first demonstration that animals can be vaccinated in a simple, painless, and economical manner by topical application of genetic vectors onto the skin. This strategy may allow the development of vaccines that could be administered by individuals without specialized medical training or equipment.

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Seeing where your hands are

Some patients with brain damage fail to identify a sensory stimulus presented on the opposite side to their lesion (contralateral) when a competing stimulus is presented on the same side (ipsilateral)¹. This phenomenon has become known as extinction. It is commonly studied using a single sense such as sight or touch (unimodal extinction)². We have studied a 75-year-old right-handed man (patient GS) who has severe left tactile extinction resulting from damage to the right frontotemporal cortex caused by a stroke. We found that an ipsilateral visual stimulus could induce extinction of a contralateral tactile stimulus (cross-modal extinction). We also found that the visual stimulus operates in a reference system attached to the hand, and not in egocentric coordinates (that is retinal, head or trunk-centred coordinates).

We tested the extinction phenomenon under six experimental conditions. Patient GS sat at a table opposite the experimenter, with his hands positioned on the table surface. In condition 1, tactile stimuli were applied to one or both hands which were placed beneath a cardboard shield to prevent him from viewing them directly. Each stimulus consisted of a light touch to the third finger. In condition 2, the shields were removed and the experimenter applied visual stimuli just in front of GS's third fingers (the same movement as the touch). GS was asked to say how many stimuli he had detected. In both conditions, he reported unilateral stimuli without errors. On stimulation of both sides simultaneously, GS showed marked tactile (1/30 correct trials) but not visual extinction.

In condition 3, GS's left hand was screened with the shield, whereas his right hand was in his view. A tactile stimulus was given to the left hand and a visual stimulus near to the right hand. On single-stimulus trials, his performance was flawless. In contrast, on double-stimulus trials, GS showed a severe left tactile extinction (0/30 correct).

Condition 4 was like condition 3, except that GS's right hand was placed behind his back to allow us to assess whether a visual stimulus presented at the same visual field location as in condition 3, but far from the ipsilateral hand, also produces a cross-modal extinction. In both single and double stimulus trials, GS performed correctly.

Condition 5 was like condition 3 except that the visual stimulus was presented far above the right hand, at the level of patient's eye. Again, GS's performance was faultless. Visuotactile extinction therefore manifested itself only when the visual stimulus was pre-

sented in an area immediately adjacent to the ipsilateral hand, and disappeared when it was far from the hand.

Condition 6 was like condition 3, except that GS's hands were crossed. He performed normally in single-stimulus trials, but in double-stimulus trials, he showed a severe extinction of the tactile stimuli (1/30 correct) applied to left hand (in right hemisphere). Extinction is not modulated by the positions of the hands in space.

Our findings may be explained by referring to the activity of bimodal, visuotactile neurons in the premotor cortex which have receptive fields attached to some relevant body parts^{3–6}. Some of these neurons have tactile receptive fields on the hand and corresponding visual receptive fields that extend outward from the tactile field into the space near the hand. As a consequence, activation of these bimodal neurons by a visual stimulus delivered near the hand also activates the corresponding perceptual representation of the hand. Here the simultaneous activation of the somatosensory representation of the left hand (by a tactile stimulus) and of the right hand (by a visual stimulus) produces an extinction of those stimuli presented in the weaker representation, in this case that of the left-hand.

Extinction phenomena (as well as neglect) occur when there is competition between two^{7,8} or more neural representations⁹. In addition, single-neuron studies have shown that visuotactile premotor neurons do not respond when visual stimuli are presented far from the tactile receptive field. This may explain the absence of visuotactile extinction found here and in a previous study¹⁰, when the visual stimulus was presented far from the ipsilateral hand. Our findings confirm the hypothesis that near space is coded in body-part-centered coordinates, and demonstrate the modular nature of human visual space.

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New narratives of creation

Life: An Unauthorized Biography

by Richard Fortey

HarperCollins: 1997. Pp. 399. £20

Andrew H. Knoll

In his brief poem "When I heard the learn'd astronomer", Walt Whitman recounts an evening spent at a scientific lecture. Facts and figures swirl about the hall, oppressively weighting the air, until he is compelled to flee into the night and restore his spirit beneath the canopy of stars. Although written more than a century ago, Whitman's verse resonates with a surprisingly large contemporary readership. Earlier attempts to understand the Universe and our place in it distilled nature's mystery into powerful narrative; science replaces awe with statistics.

This is a maddening accusation, and many readers of *Nature* would counter it by insisting that knowledge promotes wonder as effectively as ignorance. That may be, but scientists seldom do a good job of demonstrating the point. Current texts on the history of life might even be introduced as evidence in support of Whitman's view. The successive dominants of global ecosystems are generally recounted like the generations of Abraham in prose that instructs but rarely inspires. What a pleasure, then, to read Richard Fortey's elegant new book. Fortey, a distinguished palaeontologist at the Natural History Museum, London, authoritatively serves up the facts that lend substance to scientific debate about evolution, but in doing so he never loses sight of the fact that the evolutionary history assembled from fossils and comparative biology remains a story, and a darn good one at that. The result is the best account of life's history that I know, an engaging narrative that succeeds as literature as well as science.

Conventionally, Fortey begins with Darwin's warm little pond (or hot little hydrothermal vent) and ends with the rise of *Homo sapiens*. In between, there are illuminating discussions of Precambrian microorganisms, the Cambrian explosion, the great radiations and extinctions, dinosaurs, and more. Fortey acknowledges that the Earth itself plays an important role in this drama, as continents shift and climates fluctuate for better or worse. His focus, however, is squarely on character development — the trilobites, eurypterids and ammonites that populate his text emerge as organisms that lived and breathed rather than merely as the skeletal signposts of a lost world.

Not surprisingly, Fortey is best on his home turf of Palaeozoic marine animals; however, this history is ecumenical, addressing terrestrial as well as marine evo-

lution, plants as well as animals. My only serious complaint arises from Fortey's inextricable conclusion that competition was introduced into ecology with the Cambrian radiation of marine animals. Surely, microorganisms compete for resources (remember Gause?) and did so for billions of years before jellyfish and arthropods joined the fray? What animals added was macrophagous predation, enabling them to construct complex food webs that form a crown, intricate and unnecessary, atop ecosystems fundamentally maintained by microbial metabolism.

As good as Fortey's history is, it is nearly eclipsed by a second narrative woven contrapuntally about the first. Fortey's subsidiary theme concerns palaeontology as a way of knowing, illustrated honestly, and sometimes hilariously, by scenes from the life of Richard Fortey, palaeontologist. (His two-page sketch on the hazards of runny noses in Thailand is priceless.) Like Fortey, I came of age professionally piloting Cambridge University's little boat *Salterella* through the fjords of Spitsbergen, and I, too, harbour a secret envy of colleagues who travel to the Caribbean while I swat mosquitoes in Siberia. Thus, like many another palaeontologist, I can identify with many of Fortey's vignettes, whether they describe the sociology of field parties (deadly accurate) or the exhilaration of cracking open a

piece of limestone to find a fossil never before observed by humans.

Fortunately, you don't need to know the Latin names of Palaeozoic brachiopods to enjoy Fortey's sketches. They are drawn so deftly that anyone with a modicum of imagination will gain a vicarious appreciation of the life of the palaeontologist. Only rarely do our custodians of the past save the Ark of the Covenant from Nazis, stop evil geniuses from reconstituting dinosaurs, or even stump for punctuated equilibrium. Most of us spend our days in tedium and puzzlement poring over ancient shells, bones or leaves, glad for the occasional refreshment afforded by insight. There is generosity and jealousy among colleagues, and, in the field, a little romance, a lot of drudgery and occasionally even fear. Fortey captures it well.

Famous palaeontologists past and present make brief appearances in the text. Some are brilliant (Charles Lapworth, father of the Ordovician period, is a particular favourite), others merely eccentric (Rousseau Flower smoked cigarettes in the shower and lashed barren outcrops with a bullwhip). There are even a few snakes and scallywags. These characters provide incontrovertible evidence that the reconstruction of evolutionary history is, like other branches of science, a very human endeavour. Only occasionally do Fortey's characterizations yield to pop psychology, as when



It's a colourful world

An iridescent moth wing is a thing of fleeting beauty, as most insects do not retain their iridescent colours for long after death. This is just one of the many observations about pigmentation that can be

found in *Color in Nature* by Penelope A. Farrant (Sterling, \$35, £25). With 300 photographs, it is a beautiful guide to the forms and the function of colour in the natural world.

palaeontological resentment about the impact theory of end-Cretaceous extinction is ascribed to an irrational fear of apocalypse, or when parents applauding their baby's first steps subconsciously celebrate the rise of bipedal primates. Given the generosity of the text in general, Fortey's portrayal of research conferences strikes a sour and dissonant note.

Perhaps the greatest pleasure of reading Fortey's book is provided by the prose itself. His style is conversational, literate, and relaxed — Darwin as told to Calvin Trillin. A page that begins with the biology of sponges might proceed to a rumination on the use of animal names as insults (see "snakes" above) and end with an attempt to rehabilitate the epithet "slime". Patience is rewarded — as often as not Fortey's digressions fold back on the main narrative to reveal it from a new perspective.

In making the case for the history of life as science's creation narrative, and palaeontology as a way of understanding how we got here, Fortey effectively counters Whitman's accusation that scientists rob nature of joy and wonder. But then, even Whitman was capable of proclaiming: "Of physiology from top to toe I sing.... Of life immense in passion, pulse, and power". Richard Fortey could hardly disagree. □

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Fabric of the Universe

Shadow of a Star: The Neutrino Story of Supernova 1987A

by Alfred K. Mann

W. H. Freeman: 1997. Pp. 210. \$22.95, £16.95

Cosmic Clouds: Birth, Death, and Recycling in the Galaxy

by James B. Kaler

W. H. Freeman/Scientific American Library: 1997. Pp. 252. \$32.95, £22.95

F.D. Kahn

Supernova 1987A is the most famous supernova of them all. There are two main reasons: it was the first supernova visible to the naked eye for some four centuries and it was the first ever to be detected by its neutrino flux. In fact the neutrinos were registered around three hours ahead of the first optical detection — not surprising really, because the neutrinos are produced deep down within the star that became a supernova and travel straight out from there. The light from the event, however, is not emitted until the shock wave reaches the surface of the precursor star; the passage from the deep interior took three hours in the case of 1987A. In all, 20 neutrinos were collected at the Kamioka and the Lake Erie installations,

carrying between them about 10^{-10} joules, or roughly one part in 10^{56} of the energy released in the collapse of the precursor to a neutron star.

What a fantastic success; and the experiment had not even been planned that way. As Alfred K. Mann explains in his delightful book, the apparatus was designed to detect neutrinos emitted during the putative decay of protons. One tends to think of the proton as a stable constituent of the Universe, but theory has it that even protons do not last forever, with a lifetime possibly as short as 10^{30} years. In the event, this turned out to be an underestimate, and so the apparatus was left with nothing to look at. Mann tells the story of how he and his colleagues modified their experiment, what incredible care had to be taken with their procedures and how they made their great coup just months after the final adjustments. Those 20 neutrinos are probably the most important particles in the history of astrophysics, and will continue to be so until someone actually catches a magnetic monopole. But the biggest surprise of all is that nobody on the Kamiokande team has yet been awarded a Nobel prize. If ever recognition was overdue, here is a prime example.

Mann's book is compact, and focused on one essential aspect of astrophysics. By contrast, James B. Kaler's book is large and wide-ranging, although a little small for a coffee table. It is concerned with great theories, and all astrophysical life is there. The illustrations vie with each other in magnificence. Inevitably, the most striking of them are pictures of diffuse objects such as the Eagle Nebula, a region of active star formation in interstellar space, and of the Helix Nebula, a planetary nebula. Both photographs were taken with the Hubble Space Telescope — where would we be without it?

Kaler has organized his text around the pictures to trace the sequence of events whereby material in space takes its various known forms: how we can observe it and what we need to do to understand its physical and dynamical evolution. It is a bold scheme, but perhaps the author asks too much of his audience. It is hard to expect readers to spend great lengths of time ploughing through, say, the ins and outs of molecular spectroscopy when they could be feasting their eyes on all the gorgeous colour pictures. And quite right, too: as the Bard says, in *Love's Labour's Lost*: "Small have continual plodders ever won save base authority from others' books."

Both Kaler's and Mann's books illustrate one great eternal truth. If an author knows what he wants to say then his text is easy to read. When Kaler deals with the subject of planetary nebulae or when Mann describes his experiences with Kamiokande, then the reader is carried along willy-nilly, like a yacht with a following wind. But the going gets much rougher when they write about sub-



Watching the neutrinos

Supernova 1987A appears in the photograph on the right, taken a few days after the explosion, as a bright spot in the upper right where before there was none. The pictures are reproduced in *A Short History of the Universe* by Joseph Silk, which is now out in paperback. When first published in 1994, it was described by Michael

Rowan-Robinson in these pages as "the best introduction to cosmology for the general reader currently available". The new edition comments on improved images from the Hubble Space Telescope and recent searches for dark matter. W. H. Freeman/Scientific American Library, \$19.95, £14.95.

jects outside their ken, as witness Kaler's strange views on barred galaxies or on synchrotron radiation. And Mann, for all his wisdom, delivers himself of the following insight: "the supernova phenomenon... is an event with far more violence than humans can produce, but unlike much of our violence it is not mindless". Something inside one boggles. A learned rabbi once told me that no human construct should ever be expected to be perfect, and that only the Almighty can achieve perfection. He was probably right. □

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Tomato-watching in a dark corner

Readings on Color: Volume 1 – The Philosophy of Color; Volume 2 – The Science of Color

edited by Alex Byrne and David R. Hilbert
MIT Press: 1997. Pp. 317/465. \$50, £42.50 (hbk), \$30, £25.50 (pbk) each

Michael Morgan

Are tomatoes red in the dark? If your interest in this question was extinguished by late-night discussions with your undergraduate friends, read this review no further. Otherwise, open Volume 1 of this book and meet "Mary the super-scientist" who doesn't know what it's like to see red; the "Invert" who sees tomatoes as blue; and — for all I know, since I skipped a few pages — Superman, with his useful ability to see colours under short-wave-length illumination.

Both volumes are aimed, it seems, primarily at philosophers. The editors are philosophers at the Massachusetts Institute of Technology and the University of Illinois.

I learned from a recent radio broadcast by the UK geneticist Steve Jones that London's Albert Hall, home of the Promenade Concerts, was originally intended as part of the 'Albertopolis project' that would unite the sciences and the arts, following the successful Exhibition of 1851. Alas, all that resulted from Prince Albert's grand scheme was an exhibition of Bulgarian wine in the Albert Hall.

When asked by Jones to account for the failure of the Prince Consort's project to unite the sciences and the arts, the present secretary to the commissioners for the 1851 Exhibition, Patrick Middleton, observed, interestingly, that he blamed the philosophers. Philosophers, he said, are supposed to keep knowledge from fragmenting. Instead, they have retreated into a dark little corner of their own, in which philosophers are experts on philosophy. No more contemplation of the starry heavens above and the moral law within, thank you very much.

Alex Byrne and David R. Hilbert deserve credit for joining the 'neurophilosophers' who are trying to bring philosophers back to science. They clearly believe that philosophers who want to study colour should know some colour science.

Volume 1 shows how desperately this reform is needed. Let us join the philosophers in considering the unilluminated tomato. Is the tomato a fruit or a vegetable? No, sorry, that's a different problem — I mean is it red in the dark? I take it that no colour scientist would disagree with the following.

Of course, we do not see the tomato as red in the dark, and will not do so until transgenics offers us a combination of the tomato and the deep-sea fish. But the tomato in the dark has a surface reflectance spectrum that causes it, in daylight, to look red to us. The light that reaches our eye is a mathematical product of the surface reflectance spectrum of the tomato and the spectrum of the illuminant — say, northern daylight. The tomato continues to look red when illuminated by sunlight bouncing off green leaves. But this colour constancy has its limits. The tomato does not look red under sodium streetlights, and it most emphatically does not look red in the dark.

Whether one says tomatoes are red in themselves or not would seem to most scientists to be a fairly uninteresting question for the attention of lexicographers. But if you are a certain kind of philosopher, you take sides on the question and fight your corner.

You claim, like J. J. C. Smart, that colours are properties of objects, and you are a physicalist; or you say they are properties of objects that cause colours to be seen, and you are a dispositionalist; or you adhere to eliminativism and say that colours are illusions. Or, if you are a sensible sort of chap — and they nearly all are chaps — you say there is merit in all these positions. The economic advantages of a system in which everyone is paid to take in everybody else's washing are thereby convincingly demonstrated.

Most of the articles reprinted in *The Philosophy of Color* rehash the physicalist versus dispositionalist versus eliminativist positions until one groans with boredom. Having once put the book down, I had great difficulty in picking it up again. Why do so many philosophers prefer thought-experiments to real ones?

For example, we read in one chapter: "Surely, people can see things as red without even having the concept of a tomato or a [British] phone booth". But could one in fact see red without any concept of red objects: if one had never associated warmth with the red Sun seen through closed eyelids? Probably, but I see no "surely" about it.

To be fair, some chapters, and particularly the one by the editors themselves, do deal with recent discoveries about the properties of nat-

urally occurring surface reflectance spectra. And Justin Brookes contributes an interesting chapter, describing the colours that emerge from flickering achromatic stimuli in Benham's top, and the doomed Butterworth encoder which tried to use this as a basis for colour television.

But elsewhere it is all Mary the super-scientist and her relatives. Why don't Mary's friends consider instead the far more interesting cases of real people with defective colour vision in one eye, who are described nicely in Volume 2?

Also, to be fair, scientists play some part in causing confusion. Newton's statement that the "Rays are not coloured" is well known. Galileo is quoted on page 81: "Hence I think that these tastes, odours, colours etc. are nothing else but mere names." I recall a public lecture by an eminent colour scientist entitled "Colour: The Great Illusion". If colour is an illusion, then so too is the movement seen in the cinema or television. But it is pointless to argue about an issue that is merely terminological.

Let us return to the science. The chapters in Volume 2 are mostly reprinted from scientific journals. We learn from the introduction that the selections were chosen "with an eye to their philosophical relevance". This rang alarm bells for me. All advances in colour science are of philosophical relevance if philosophy is to be understood in the generous spirit of the chairman of the commissioners for the 1851 Exhibition. It is a cop-out to suggest to philosophers that they can pick the plums out of the literature without being troubled by the details.

Many solid scientific papers are here, to be sure, but it is scarcely believable that the most recent paper on the neural coding of colour is from 1977! The term "cardinal axis" figures neither in the helpful glossary nor in the index. The key question of how the achromatic and chromatic signals are disentangled from the univariant parvocellular pathway is nowhere to be found: the reader has to make do with a paper from 1982.

To call this collection heteroclitic would be to exaggerate its cohesiveness. The editors do not seem to have made up their minds whether to present a volume of classic papers or one of recent advances. Physiology, as I have indicated, is particularly badly served. On the other hand, molecular genetics is well represented by a *Scientific American* article by Jeremy Nathans, and there is welcome attention paid to the ecology and evolution of colour vision. The chapters on the physics and chemistry of surfaces will be useful to many colour scientists.

Interestingly, what is completely lacking is anything serious on the aesthetics of colour. Could not painters claim to be the true experts on colour science? The Albert Hall lies only a few minutes' walk from London's Imperial College of Science, Technology and Medicine,

but this is still too far, it seems, for modern philosophers. □

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Related book

Do visual science and anthropological linguistics have anything to say to each other? Does the make-up of our colour-vision system constrain our linguistic expression of colour categories? Does the way we use colour language suggest anything about the biological organization of colour vision? These are the questions addressed in *Color Categories in Thought and Language* edited by C. L. Hardin and Luisa Maffi (Cambridge University Press, £55, \$74.95 (hbk), £19.95, \$27.95 (pbk)), which unfortunately arrived too late for inclusion in Michael Morgan's review above. The book arose from a 1992 conference which brought together visual scientists, psychologists, linguists and anthropologists to examine claims that commonalities of basic colour term use extend across languages and cultures and probably express universal features of perception and cognition.

Mind over chatter

The Symbolic Species: The Co-Evolution of Language and the Brain

by Terrence Deacon

Norton/Allen Lane: 1997. Pp. 496. \$27.50, £20

David Poeppel

One of the troubling things about studying language is that almost everyone is willing to contribute an opinion on how language works, including scientists from other fields who should know better. This is not typically true when one studies, say, the kidney or motor control. Language is viewed as an area that licenses unconstrained speculation. Despite all lay intuitions, however, the scientific study of language requires technical expertise.

Terrence Deacon's *The Symbolic Species* exemplifies how an ambitious book about the coevolution of language and the brain fails because one half of the question (how language works) is treated in an outdated and superficial way. The author aims for a theory of language but develops a theory of everything, ranging from word meaning to empathy to consciousness. I, for one, am invariably suspicious of theories of everything.

In modern cognitive science, neuroscience and psychology, two strongly opposed positions about language dominate the agenda. One position is that acquiring, knowing and using language all reflect the existence of a specialized cognitive system or 'language faculty'. In this view, language con-

stitutes an autonomous mental domain, independent of other cognitive abilities such as face recognition or inductive reasoning. This framework was most forcefully developed by Noam Chomsky and has recently received a popular exposition in Steven Pinker's *The Language Instinct* (Morrow/Allen Lane, 1994). An important assumption of the domain-specific framework is that the ability to learn language is innately specified. In today's climate in cognitive science and neuroscience, Chomsky, Pinker and other researchers with nativist inclinations are often considered the 'bad guys'.

The 'good guys' (who I believe are wrong) argue that it is unnecessary to posit language-specific innate machinery. Instead, general learning and developmental mechanisms designed to constrain learners provide sufficient explanatory infrastructure to account for the phenomena of language. A recent empiricist manifesto of this sort is J. Elman *et al.*'s *Rethinking Innateness* (MIT Press, 1996).

The position endorsed by Deacon is clear: "No innate rules, no innate general principles, no innate symbolic categories can be built in by evolution". Perhaps this shows a poverty of the imagination. Deacon arrives at this pronouncement from a comparative and evolutionary perspective. What does this mean? First, that he compares animals and humans. Second, that he wants to explain the coevolution of language and the brain in a way that maintains the continuity of brain evolution in the face of the discontinuity in behaviour: humans have language, animals do not. He is particularly impressed with the communicative abilities of Kanzi, the chimpanzee trained by Sue Savage-Rumbaugh, who offers a "devastating challenge to the nativist, Chomskyan perspective". Precisely what that challenge amounts to is not mentioned. How a chimp can challenge a linguistic theory is still not clear to me.

Deacon's argument proceeds in three stages. He lays out what he considers to be the core of language (symbolic reference); works through the neurological ingredients that support his framework (the relative increase in the size of the prefrontal cortex); and presents his story of how language and the brain coevolved.

The central notion of what constitutes language for Deacon is symbolic reference. His conceptualization of this underlies the entire comparative and evolutionary story. Insofar as one is sceptical, the story becomes problematic. Drawing on the ideas of C. S. Peirce, Deacon argues that reference, the relation between words and the world, is iconic (based on the similarity between the object and the referring signifier), indexical (based on spatial or temporal contingencies) or symbolic. Humans alone are specialized for symbolic reference, and features such as knowledge of syntax are consequences of symbolic reference. Why are humans

human? Not because they are hairless apes with language (the generative straw man) but because they have crossed a mystical "symbolic threshold", Deacon contends.

Much of this discussion is unsatisfying because Deacon is out of touch with research on language. For example, he constantly uses the expression "grammar and syntax" (both terms have technical definitions; Deacon defines neither); he seriously misinterprets the term "deep structure" (which has precise meaning in linguistics); and the view of language acquisition that he attributes to generative linguistics implies that research halted 30 years ago.

Deacon is at his best discussing neuroscience. His breadth of knowledge is impressive throughout; his description of the neural basis of vocalization is especially clear. It is central to his argumentation that the relative increase in the size of the prefrontal cortex in humans enlarged working memory and attentional capacity and made possible the better manipulation of symbolic representations.

Again, though, technical inaccuracies weaken the presentation. The brief discussion of neuroimaging, a field that is contributing considerably to our understanding of the neural basis of language, is out of date. In his discussion of Williams' syndrome, Deacon makes a lot out of preserved prefrontal cortex and cerebellum in the face of significant reductions in "posterior cortical systems". But the brain area typically associated with language processing (the planum temporale) turns out to be bigger, relative to overall brain size, in people with this condition.

Occasional stylistic excesses also do not help: "We are not just a species that uses symbols. The symbolic universe has ensnared us in an inescapable web. Like a 'mind virus,' the symbolic adaptation has infected us, and now, by virtue of the irresistible urge it has instilled in us to turn everything we encounter and everyone we meet into symbols, we have become the means by which it unceremoniously propagates itself throughout the world".

When one makes big claims about how language works, one needs to show something about language: explicit, worked-out examples. A general discussion of what a theory might do does not suffice. Plainly, so expansive a book owes the reader more.

Who will like this book? Anyone who is already persuaded by the positions of Elman, Bates, Seidenberg and other anti-nativists. Those looking for a detailed explanation of language phenomena, within a framework that goes beyond speculation, will be disappointed. The 'bad guys' are still badder. □

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Hydrothermal syntheses and structural characterization of zeolite analogue compounds based on cobalt phosphate

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Zeolite analogues containing transition metals are highly desirable for industrial processes. A generalized synthesis method has been developed and demonstrated to have the potential to generate many transition-metal-rich zeolite-type structures. The method has been used to make zeolite analogues based on cobalt phosphate with diverse chemical compositions and structure types, including some analogues never previously synthesized and some zeolite-like structures only previously known theoretically. The concentrations of transition-metal atoms in the frameworks can be controlled by varying the charge and geometry of the structure-directing amine molecules.

Zeolites have been extensively studied because of their utility in industrial processes such as catalysis and gas separation¹. Traditionally, both natural and synthetic zeolites are aluminosilicates. Recently, zeolite analogues and novel microporous structures based on aluminophosphates², substituted aluminophosphates^{3,4}, and zinc- (or beryll- or) phosphates (or arsenates)^{5,6}, have been reported. Tetrahedrally coordinated atoms in some of these structures can be substituted by other elements, which gives rise to other families of structures such as the gallophosphates^{7,8}.

There have been many efforts to synthesize transition-metal-containing zeolite analogues because of their potential importance in industrial catalysis⁹. Incorporation of transition metals has been successful in a number of systems, particularly in the aluminophosphate-based family⁹. However, the percentage of transition-metal substitution is limited and there are no examples that we are aware of in which the transition metal:aluminium ratio in the framework has been unambiguously characterized to exceed 1:1 for a zeolite analogue structure^{4,9}. In fact, no more than 38% of the Al sites have been replaced with transition-metal atoms in aluminophosphate-based zeolite structure analogues. CoAPO-44, a chabazite analogue, is one example in which 25% of Al sites (Co/Al = 0.33) are replaced with cobalt cations¹⁰. In CoAPO-50 (ref. 10), a novel aluminophosphate-based structure, 37.5% of Al sites (Co/Al = 0.6) are replaced whereas in CoAPO-46 (ref. 11) approximately 26% of Al sites (Co/Al = 0.35) are substituted. Thus despite many years of efforts, no materials with transition metals occupying the majority of the framework metal atom sites (that is, Co/Al > 1) are known to be isotopic to a mineral zeolite¹². We note that transition-metal silicates and phosphates with open-framework structures are well known. For example, one novel open-framework cobalt phosphate¹³ and numerous amine-containing vanadium phosphates have been reported¹⁴. None of these, however, are zeolite analogues, and transition metals in these structures often have non-tetrahedral coordinations.

We are interested in microporous structures built from novel polyhedral centres with different charge combinations and geometries¹⁵ and have synthesized a number of new zeolite structural analogues in the T^{2+}/T^{5+} system⁵ (here T refers to tetrahedrally coordinated atoms) in addition to a variety of novel open-framework structures^{16,17}. We find that zeolite analogue structures in the T^{2+}/T^{5+} system are formed with hydrated inorganic cations⁵ such as Li^+ and Na^+ and that the use of organic amines usually gives open-

framework structures with interruptions on either T^{2+} or T^{5+} sites¹⁶. So far no zeolite structural analogues containing only amines as guest species have been found in the T^{2+}/T^{5+} system. The difficulty in synthesizing amine-containing zeolite analogues in the T^{2+}/T^{5+} system can be understood from the host-guest charge mismatch. In reporting two sodalite-like zincophosphates¹⁶, we showed that their framework interruptions are a result of the charge matching between the frameworks and the guest molecules.

In the phosphate system, there is a strict alternation of metal- and phosphorus-oxygen tetrahedra if a zeolite analogue structure is to be formed. For the binary phosphate system, the negative charge of the framework is completely determined by a particular framework topology whereas in the silicate system, the Si/T^{n+} ($n = 2, 3$) ratio can be varied or self-adjusted to fine-tune the charge density of the framework. In the T^{2+}/T^{5+} system, the topology-determined charge density can only be balanced with the inclusion of hydrated inorganic cations in some zeolite analogues⁵ and with NH_4^+ , Rb^+ and so forth in the anhydrous synthetic ABW phases¹⁸ (here ABW refers to a type of zeolite topology). In general, the framework charge is too negative to be balanced by the positive charge of most (protonated) amines. Thus, dense frameworks or open-framework structures with interruptions are usually formed when amines are used as structure-directing agents in the T^{2+}/T^{5+} system^{19,20}.

In developing a methodology to synthesize transition-metal-based zeolite analogue structures, the cobalt phosphate system is attractive because the ligand-field stabilization energies for Co^{2+} (d^7) disfavour the tetrahedral configuration relative to the octahedral one to a lesser extent than for most other d^n configurations. A systematic examination of the simple alkali-metal and alkaline-earth-metal cobalt phosphates in relation to silica-type structures also suggests that the synthesis of cobalt-phosphate-based zeolite analogue structures should be feasible^{21,22}. However, the traditional methods of using hydrated group I and II cations in the synthesis of low-Si/Al molecular sieves are in general not applicable to transition-metal-based (that is Co^{2+}) structures with a similar framework charge density because transition metals usually have a strong interaction with H_2O and compete with alkali-metal or alkaline-earth metals for H_2O coordination. Thus, except for NH_4CoPO_4 and $RbCoPO_4$ which have the synthetic ABW type topology, no other zeolite analogues have been found in the pure cobalt phosphate system²¹.

Conventional amine-containing aluminophosphate (including

substituted aluminophosphate) and aluminosilicate structures usually have low framework charge densities and are commonly synthesized using amines with a low charge/volume ratio (sometimes quaternary amines). In extreme cases, the framework is neutral. The introduction of metal atoms into these systems is often intended to create negative charges in an otherwise neutral framework and subsequently generate catalytic sites. Aluminophosphate-based materials were discovered¹ following extensive research on molecular sieves with high Si/Al ratios, which popularized the use of amines with a low charge/volume ratio. Thus, the synthesis approach in aluminophosphate-based materials has been closely related to that used in the high-Si/Al zeolites. Ternary zeolite-type structures have been extensively studied and numerous metal-substituted aluminophosphates have been discovered, with a limitation that the M^{2+}/Al^{3+} ratio rarely exceeds 1. In terms of framework charge density, the average charge per tetrahedral atom in these metal aluminophosphates is between 3.75 and 4, similar to that for aluminosilicate molecular sieves with $Si/Al \geq 3$.

We sought to develop a synthesis method applicable to a variety of chemical compositions, including transition metals. In particular, it is desirable to have a family of T^{2+}/T^{5+} based zeolite analogues that are closely related to low-Si/Al molecular sieves ($Si/Al \leq 3$). This family of zeolite analogues would have an average charge per tetrahedral atom in the region of 3.5 to 3.75 and can involve zeolite analogues of both main-group (such as Al-Be-P-O and Al-Zn-P-O) and transition metal (such as Al-Co-P-O and Co-Co-P-O) atoms.

Compared to the amine-containing aluminophosphate and aluminosilicate structures reported previously, the divalent-metal (such as Co^{2+}) phosphate framework is highly negatively charged. The introduction of T^{3+} atoms, such as Al^{3+} and Ga^{3+} , into T^{2+} sites reduces the framework charge and gives us the ability to adjust the framework charge of a non-silicate framework structure for a given topology type. The resulting framework, rich in divalent metal atoms, is still highly charged, and amines with a high charge/volume ratio (usually a low C/N ratio) are typically employed in our syntheses. The final framework composition (that is, the Co/Al ratio) is in general determined by the shape and charge density of organic amines, and is not directly related to the initial gel composition, which is often manipulated for the optimum pH and crystal growth conditions. We note that in the synthesis of

zeolite-like structures, much attention has been focused on the structure-directing effect of the size and shape of organic amines²³ whereas charge-density matching, on which we have focused, has received less attention²⁴.

Overview of synthesis and products

Large amines can be used as a source for smaller amines owing to the hydrolysis of amines in the reaction mixture. For example, the gismondine framework with the *Fddd* space group symmetry can be formed from large amines including N,N,N',N'',N'''-pentamethyldiethylenetriamine $[((CH_3)_2NCH_2CH_2)_2NCH_3]$. The formation of ACP-CHA1 and ACP-GIS1 (Table 1) may also involve the hydrolysis of the initial amines.

All new cobalt phosphates reported here have a vivid blue colour consistent with the tetrahedral coordination of Co^{2+} . Owing to the availability of single crystals, we have been able to obtain detailed structural information on these new zeolite analogues, with the framework tetrahedral atoms and bridging oxygen atoms of all structures located and refined. In some cases, the position or the identity of the guest species are not unambiguously determined by single-crystal structure determination. More extensive studies by low-temperature X-ray diffraction and NMR spectroscopy are in progress. Some structures have been determined with very small crystals. For example, the maximum dimensions for ACP-CHA1 and ACP-SOD2 are 25 and 33 μm , respectively. The *R* factors listed in Table 1 show a large variation depending on the crystal size (ranging from 20 to more than 500 μm) and the ordering of the guest species.

In the analcime and faujasite analogues, Co, Al and P occupy the same site as there is only one unique T-atom site. In ACP-FL2, a feldspar analogue, the distribution of Co, Al and P is also random even though two unique T-atom sites are available. A completely random distribution of Al, Co and P sites is unlikely, however, and the apparent disorder observed in these three structures may be due to the presence of different crystal domains in which the metal-atom sites and P sites are exchanged. Single crystals have been obtained in which the exchange of T-atom sites is observed. For example, the P and Al(Co) sites in CAP-CHA2 and CAP-CHA2' are exchanged.

In general, for Al-containing cobalt phosphates, tetrahedral Co and Al atoms occupy the same sites that alternate with ordered P

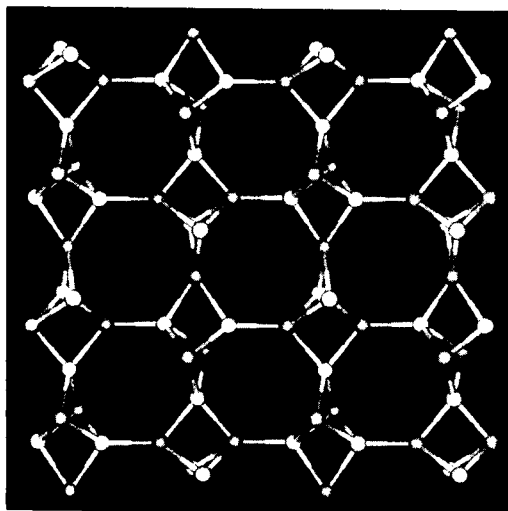


Figure 1 The structure along the *a*-axis, an infinite sheet formed from the linking of CoO_4 and PO_4 tetrahedra. The red represents the phosphorus sites; the yellow represents the cobalt (aluminum) sites.

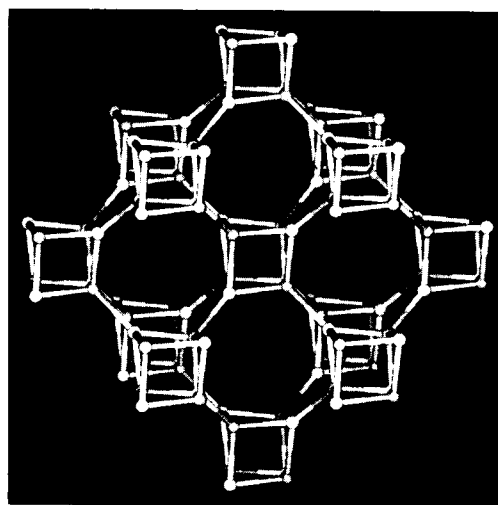


Figure 2 The tetrahedral atom connectivity diagram showing the body-centred cubic arrangement of the double 4-ring units in ACP-1. The red represents the phosphorus sites; the yellow represents the cobalt (aluminum) sites.

sites even though there is strong crystallographic evidence of pure Co^{2+} sites in thomsonite analogues and pure Co^{2+} and Al^{3+} in some other structures. Determining the T^{2+}/T^{3+} ratio is one of the most important aspects in the structural characterizations of this new family of zeolite analogues.

The average value of the T^{2+}/T^{3+} ratio in the unit cell can be derived in two different ways: (1) charge neutrality if the guest amine molecules are unambiguously determined; (2) electron-probe microanalysis, thermogravimetric analysis, and other analytical techniques if the guest molecules are not well determined. Determining the T^{2+}/T^{3+} ratio for each individual tetrahedral site is more difficult than determining its average value in the unit cell, especially for compounds with mixed $\text{Zn}^{2+}/\text{Ga}^{3+}$ or $\text{Co}^{2+}/\text{Ga}^{3+}$ sites. However, for $\text{Co}^{2+}/\text{Al}^{3+}$ sites, because there is a significant difference

between the X-ray scattering factors of Co and Al, the Co/Al ratio can be obtained from the refinement of the occupancy factors.

An indirect and often more reliable way of evaluating the Co/Al ratio for each individual Co/Al site is through the metal–oxygen bond distance. As Co–O and Al–O bond distances differ significantly (approximately 1.93 Å for Co–O and 1.74 Å for Al–O)²⁵, a bond distance closer to 1.93 Å would indicate the dominance of cobalt on that site and vice versa. We have carefully examined the relationship between the occupancy factors and average bond lengths for each Co(Al) site in well-refined structures and found that the average bond length (M–O) for each metal-atom site is linearly dependent on the occupancy factor of Co (or Al). Thus for Co/Al mixed sites, to a very good accuracy (about 0.01–0.02 Å), the following equation is true for a well-refined structure: bond

Table 1 Crystallographic data for selected zeolite analogues and novel structures synthesized in this study

Name	Structural formula*	Space group	<i>a</i> (Å)	<i>b</i> (Å)	<i>c</i> (Å)	β (deg)	<i>R</i> (<i>F</i>)	$2\theta_{\text{max}}$ (deg)	M–O (Å)
ACP-ANA1	$\text{Cs}_2\text{AlCo}_2\text{P}_3\text{O}_{12}$	<i>Ia</i> 3 <i>d</i>	13.820	13.820	13.820	90	10.2	50	1.69(P)
ACP-CHA1	$\text{Al}_{10}\text{Co}_9\text{PO}_4\text{t}$	<i>R</i> 3̄	13.890	13.890	15.329	90	14.5	35	1.88
ACP-CHA2	(R5) $\text{Al}_2\text{Co}_2\text{P}_4\text{O}_{16}\cdot\text{H}_2\text{O}$	<i>R</i> 3̄	13.867	13.867	15.056	90	6.49	50	1.821
ACP-CHA3	(R6) $\text{Al}_2\text{Co}_2\text{P}_4\text{O}_{16}\cdot\text{H}_2\text{O}$	<i>R</i> 3̄	13.806	13.806	15.082	90	5.25	56	1.800
ACP-CHA4	(R17) $\text{Al}_2\text{Co}_2\text{P}_4\text{O}_{16}\cdot\text{H}_2\text{O}$	<i>R</i> 3̄	13.835	18.835	15.250	90	11.5	50	1.808
ACP-CHA5	(R61) $\text{Al}_2\text{Co}_2\text{P}_4\text{O}_{16}\cdot\text{H}_2\text{O}$	<i>R</i> 3̄	13.843	13.843	14.839	90	9.51	50	1.788
CAP-CHA1	(R14) $\text{CoAl}_2\text{P}_3\text{O}_{12}$	<i>R</i> 3̄	13.746	13.746	15.299	90	8.67	50	1.779
CAP-CHA2	(R15) $\text{CoAl}_2\text{P}_3\text{O}_{12}$	<i>R</i> 3̄	13.713	13.713	15.131	90	11.6	40	1.776
CAP-CHA2'	(R15) $\text{CoAl}_2\text{P}_3\text{O}_{12}$	<i>R</i> 3̄	13.828	13.828	15.260	90	10.7	40	1.782
ACP-GIS1	$\text{Al}_{10}\text{Co}_9\text{PO}_4\text{t}$	<i>C</i> 2/ <i>c</i>	14.392	9.422	10.015	132.76	9.73	40	1.89
ACP-GIS2	(R1) AlCoP_2O_8	<i>I</i> 2/ <i>a</i>	9.778	9.182	10.314	90.59	3.17	56	1.846
ACP-GIS3	(R11) AlCoP_2O_8	<i>C</i> 222 ₁	13.826	14.479	10.166	90	7.86	50	1.817
ACP-GIS4	(R11) AlCoP_2O_8	<i>F</i> ddd	10.220	14.145	14.601	90	5.58	50	1.816
ACP-GIS5	(R12) $\text{Co}_3\text{Al}_{1-3}\text{PO}_4$	<i>C</i> 222 ₁	14.206	14.801	9.915	90	12.5	42	1.82
ACP-GIS6	(R16) $\text{Co}_3\text{Al}_{1-3}\text{PO}_4$	<i>C</i> 222 ₁	14.234	14.770	9.843	90	9.00	45	1.789
ACP-MER1	$\text{Al}_{1-3}\text{Co}_{1-3}\text{P}_2\text{O}_8\text{t}$	<i>Ccca</i>	20.147	20.515	10.024	90	10.9	40	1.85
ACP-MER2	(R2) $\text{AlCo}_2\text{P}_4\text{O}_{16}\text{t}$	<i>P</i> 4/ <i>nn</i> c	14.662	14.662	9.615	90	7.73	50	1.861
ACP-PH1	(R32) $[\text{NH}_4]\text{AlCo}_3\text{P}_4\text{O}_{16}$	<i>C</i> 2/ <i>c</i>	10.223	14.605	14.304	90.162	7.00	50	1.857
ACP-SOD1	(R81) $\text{AlCo}_2\text{P}_3\text{O}_{12}$	<i>P</i> 4 ₂ / <i>n</i>	12.728	12.728	8.7357	90	4.94	50	1.833
GCP-SOD1	(R81) $\text{GaCo}_2\text{P}_3\text{O}_{12}$	<i>I</i> 4 ₁ / <i>a</i>	17.429	17.429	18.541	90	4.71	56	1.890
ACP-THO1	(R3) $\text{AlCo}_4\text{P}_5\text{O}_{20}$	<i>C</i> 2/ <i>c</i>	14.130	13.178	14.018	90.56	12.5	42	1.91
ACP-THO2	(R21) $\text{AlCo}_4\text{P}_5\text{O}_{20}$	<i>C</i> 2/ <i>c</i>	13.858	13.229	13.877	90.75	5.91	50	1.902
GCP-THO1	(R3) $\text{GaCo}_4\text{P}_5\text{O}_{20}$	<i>C</i> 2/ <i>c</i>	14.137	13.213	13.967	90.61	15.3	40	1.95
GCP-THO2	(R21) $\text{GaCo}_4\text{P}_5\text{O}_{20}$	<i>C</i> 2/ <i>c</i>	13.808	13.249	13.842	91.33	6.97	48	1.910
GCP-THO3	(R3) $\text{GaCo}_4\text{P}_5\text{O}_{20}$	<i>P</i> 2 ₁	9.301	14.240	9.821	95.52	15.5	38	1.93
GCP-THO4	(R3) $\text{GaCo}_4\text{P}_5\text{O}_{20}(\text{H}_2\text{O})$	<i>P</i> 2 ₁ / <i>n</i>	12.923	14.226	14.198	92.80	5.28	50	1.924
AZP-THO3	(R3) $\text{AlZn}_4\text{P}_5\text{O}_{20}$	<i>P</i> 2 ₁	9.511	14.177	9.706	94.87	14.8	40	1.93
ACP-FL1	$\text{NH}_4\text{AlCoP}_2\text{O}_8$	<i>C</i> 2/ <i>c</i>	13.404	13.205	8.935	100.89	7.17	50	1.833
ACP-FL2	$\text{NH}_4\text{AlCoP}_2\text{O}_8$	<i>C</i> 2/ <i>m</i>	8.931	13.188	7.314	115.96	4.74	50	1.67(P)
ACP-	(R2) $(\text{Al}_{0.22}\text{Co}_{1.78}\text{P}_2\text{O}_8)_x \cdot 1/2\text{H}_2\text{O}$	<i>I</i> 42 <i>m</i>	10.221	10.221	9.585	90	7.76	50	1.916
ACP-2	(R4) $(\text{NH}_4)\text{AlCo}_3\text{P}_4\text{O}_{16}$	<i>P</i> bcm	8.910	14.974	14.712	90	12.6	38	1.86
GCP-2	(R4) $(\text{NH}_4)\text{GaCo}_3\text{P}_4\text{O}_{16}$	<i>P</i> bcm	8.909	14.975	14.789	90	12.2	40	1.95
UCSE-4	(R22) $\text{Co}_2\text{Al}_4\text{P}_6\text{O}_{24}$	<i>P</i> 2 ₁ / <i>c</i>	9.259	18.880	9.512	118.08	11.9	45	1.79
CAP-AE11	(R83) $\text{Co}_2\text{Al}_4\text{P}_6\text{O}_{24}(\text{H}_2\text{O})_3$	<i>C</i> 2/ <i>c</i>	13.911	12.772	18.634	90.359	9.35	45	1.788
CAP-FAU1	$\text{Co}_{0.33}\text{Al}_{0.67}\text{PO}_4$	<i>F</i> d3 <i>m</i>	24.802	24.802	24.802	90	15.5	40	1.65(P)

Here $R(F) = \sum |F_o| - \sum |F_c| / \sum |F_o|$ with $F_o > 4.0\sigma(F)$. Single-crystal data with Mo K α . The $2\theta_{\text{max}}$ refers to the maximum 2θ value for reflections used in the structure refinement. "P" in the bond distance list indicates that Al, Co and P occupy the same site. ACP stands for aluminium cobalt phosphate.

* In formulae, fractional numbers are derived from electron probe microanalysis. $x \leq 2/3$; $0.2 \leq y \leq 0.6$; $2 \leq z \leq 3$; R1, CH_3NH_2 ; R11, $(\text{CH}_3)_2\text{NH}$; R12, $(\text{CH}_3)_2\text{CHNH}_2$; R14, $(\text{CH}_3\text{CH}_2)_2\text{N}$; R15, $(\text{HOCH}_2\text{CH}_2)_2\text{N}$; R16, $\text{CH}_3(\text{CH}_2)_2\text{NH}_2$; R17, $\text{HN}(\text{CH}_2\text{CH}_3)_2$; R2, $\text{NH}_2(\text{CH}_2)_2\text{NH}_2$; R21, $\text{CH}_3\text{NH}(\text{CH}_2)_2\text{NH}_2$; R22, $(\text{CH}_3)_2\text{N}(\text{CH}_2)_2\text{N}(\text{CH}_3)_2$; R3, $\text{NH}_2(\text{CH}_2)_2\text{N}(\text{CH}_3)_2$; R32, $\text{NH}_2\text{CH}_2\text{C}(\text{CH}_3)_2\text{CH}_2\text{NH}_2$; R4, $\text{NH}(\text{CH}_3)_2$; R5, $\text{NH}(\text{CH}_3)_2$; R6, $\text{NH}_2(\text{CH}_2)_2\text{NH}_2$; R61, *C,C,C*-trimethyl-1,6-diaminohexane; R81, piperazine; R83, 4-amino-2,2,6,6-tetramethyl-piperidine; all amines are fully protonated.

† These compounds contain unidentified guest species.

length = $1.74 + 0.19 \times \text{occupancy of cobalt}$. This relationship is useful because bond distances and occupancy factors may not have the same reliability in a non-ideal refinement. In general, occupancy factors are more likely to be correlated with other factors such as thermal parameters and are particularly unreliable when guest amines are seriously disordered and not included in the refinement. Because of the importance of bond lengths in evaluating Co/Al ratios, the average M–O distances of all unique metal-atom sites in each structure are compiled in Table 1.

For structures having indeterminate guest species, residual electron density peaks and their geometrical distributions indicate that, with rare exceptions, these peaks can only be C, N or O atoms. The additional support for the absence of extra-framework Co^{2+} atoms is provided by the electron-probe microanalysis which shows that the sum of Al and Co in atomic numbers equals that of P within experimental error.

New analogues and structures

In the following, we discuss our materials according to their structural types, with the focus on structures with $\text{Co/Al} > 1$. The crystallographic data for these structures, together with some new structures with $\text{Co/Al} \leq 1$, are listed in Table 1.

Analcime ($\text{Co/Al} = 2$). This cobalt-rich phosphate (ACP-ANA1) with the analcime type topology is isostructural with the mineral pollucite⁸. In such a structure, there is only one crystallographically unique tetrahedral atom site. As the Al–O, Co–O and P–O bond lengths are significantly different, the disorder of tetrahedral atoms affects the positional ordering of bridging oxygen atoms. The refined T–O distance is 1.69(3) Å, in agreement with the weighted average (1.69 Å) of P–O (3×1.52 Å), Co–O (2×1.93 Å) and Al–O (1×1.74 Å) bonds²⁵.

Chabazite ($\text{Co/Al} > 0.3$). Chabazite analogues have been synthesized with about two dozen different amines. Most of these amines were not previously known to be able to assist in the formation of the chabazite framework. The Co/Al ratio in the triethylamine phase is 0.5 and can be increased to 1 when 1,6-diaminohexane is used as the structure-directing agent. A further increase of Co/Al ratio is achieved with 1-(2-aminoethyl)piperazine. These results illustrate the flexibility of the chabazite framework and show that the charge/volume ratio of organic amines can be used to control the framework Co/Al ratio and its overall charge.

Sodalite ($\text{Co/Al} = 0.5, 2$). We have previously used both monoprotonated and diprotonated piperazine in the syntheses of two sodalite-like zincophosphates¹⁶. The charge matching between the framework and the guest amine in these structures is achieved through cage interruption and expansion. This is truly designed structural templating, in that at least one point-group symmetry element of the organic structure and the molecular charge determine the framework composition and geometry. The following examples of defining the transition-metal content in sodalite analogue structures by using amines with the appropriate charge and geometry in the cobalt phosphate system further illustrate the level of control that can be achieved in the syntheses of porous materials.

A cubic cobalt aluminophosphate sodalite analogue has been prepared with piperidine as the structure-directing agent. The Co/Al ratio derived from electron microprobe analysis is 0.5, in agreement with one monoprotonated piperidine per sodalite cage. A cobalt-enriched sodalite structure requires increased charge for similar organic shape and volume, and diprotonated piperazine and dabco (here dabco refers to 1,4-diazabicyclo[2.2.2]octane) were therefore used in two separate syntheses. As expected from the above line of reasoning, the resulting structures, also sodalite analogues structurally templated by diprotonated piperazine or dabco, have a Co/Al ratio of 2. As the host–guest interaction in the dabco sodalite is not as strong as that in the piperazine sodalite, dabco is orientationally disordered inside the cage so that the usual

cubic symmetry of the sodalite cage is preserved in ACP-SOD2. Among numerous known sodalite structures, these piperazine- and dabco-templated sodalite structures are the first two sodalites in which the metal-atom sites are dominated by transition-metal atoms. We note that the average charge per sodalite cage in ACP-SOD1 and ACP-SOD2 is -2 , in contrast with previously known sodalite structures which can have a charge of 0, -1 , -3 or -6 .

In ACP-SOD1 (tetragonal, space group $P4_2/n$), the sodalite cage symmetry is dictated by the point-group symmetry of the piperazine molecule in a manner similar to that found in interrupted and expanded sodalite cages¹⁶. The symmetry element that affects the cage symmetry here is the inversion centre. The host–guest interaction that allows the communication of the symmetry information from the guest molecule to the framework is hydrogen bonding between NH_2 groups and framework oxygen atoms. The two shortest N...O contacts possibly involved in the hydrogen bonding are 2.76 Å for N1–O2 and 3.05 Å for N1–O5.

Thomsonite ($\text{T}^{2+}/\text{T}^{3+} = 4$). So far no synthetic materials (even an aluminosilicate) have been structurally characterized to be isotypic to thomsonite. A gallosilicate thomsonite analogue was reported with insufficient structural data²⁶. Using two different organic amines, 1,3-diaminopropane and *N*-methylethylenediamine, we have synthesized more than half a dozen phosphates with the thomsonite topology having several different lattice symmetries. Most of these phases are based on cobalt phosphate. This is the first time (to our knowledge) that the formation of the thomsonite framework has been templated by organic amines. Here we demonstrate that organic amines with nearly the same shape and charge/volume ratio lead to the same framework charge density and topology. The average charge per tetrahedral atom in thomsonite analogues is 3.6, the lowest among zeolite or zeolite analogue structures templated with only organic amines.

Even though both 1,3-diaminopropane and *N*-methylethylenediamine can template the formation of the thomsonite framework, the location of nitrogen atoms in the 5-atom chain affects the ordering of amines inside the channel and also the crystal growth. Although it is easy to grow large crystals of *N*-methylethylenediamine-templated thomsonite structures, those templated by 1,3-diaminopropane grow into thin fibres.

Thomsonite is one of a few known fibrous zeolites, and its structure has a characteristic chain not found in non-fibrous zeolites. These chains are linked into a layer shown in Fig. 1. The repeat of such a layer along the *a*-axis creates the framework of thomsonite. We have also synthesized two phases, GCP-THO1 and GCP-THO2, which are isostructural with ACP-THO1 and ACP-THO2, respectively. The gallium cobalt phosphate thomsonite analogues are the first zeolite analogues in which the Co/Ga ratio exceeds one. A cobalt gallium phosphate analogue ($\text{Co/Ga} = 1$) of the zeolite gismondine is already known²⁷. As the ideal Ga–O (tetrahedrally coordinated Ga^{3+}) distance is 1.82 Å (ref. 25), the incorporation of Ga^{3+} onto the cobalt sites reduces the M–O bond distance. Such a reduction is obvious, but not significant, in thomsonite analogues owing to the high Co^{2+} concentration.

Gismondine ($\text{Co/Al} > 0.33$). Synthesizing a transition-metal-rich gismondine type framework is difficult, and most amines we used give a Co/Al ratio of 1. We have successfully found an organic template, tetraethylenepentamine, that can template the formation of a cobalt-rich gismondine framework. In this structure (ACP-GIS1), cobalt cations occupy about 80% of metal-atom sites.

More than half a dozen different gismondine structures in three different space groups have been obtained by using different organic amines. Most of these structures have the orthorhombic symmetry, whereas ACP-GIS1 and ACP-GIS2 are monoclinic but have pseudo-orthorhombic cells. Amines in these gismondine analogues are disordered to different extents.

In ACP-GIS2, the orientation of methylamine molecules inside

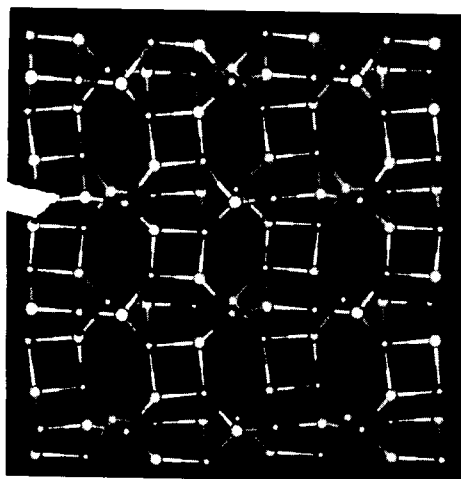


Figure 3 The 3D framework formed by linking double saw (ss) chains along the c -axis in merlinoite (ACP-2). The red represents the phosphorus sites; the yellow represents the cobalt (aluminum) sites.

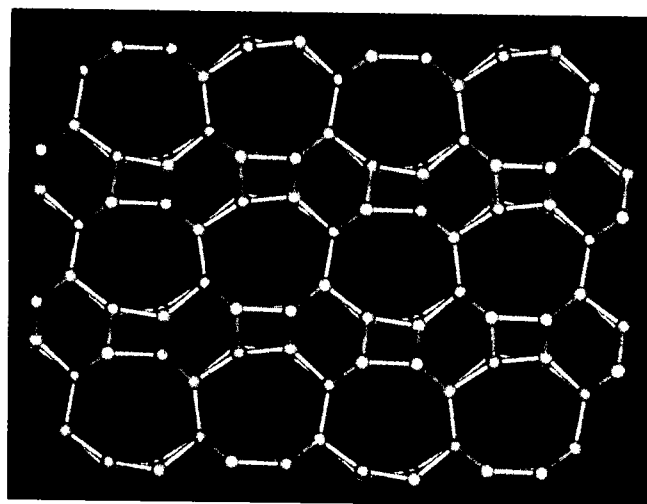


Figure 4 The 8-ring channels viewed down the c -axis in ACP-2. The red represents the phosphorus sites; the yellow represents the cobalt (aluminum) sites.

the channel affects the host–guest interaction. The monoprotonated methylamine (CH_3NH_3^+) is orientationally disordered so that the average molecular shape looks like the tetramethylammonium cation (TMA⁺). However, which atom (C or N) is at the tetrahedral centre of the average methylamine atomic sites is of importance in understanding the formation of the framework. The structural data indicate that the carbon is at the tetrahedral centre (also the centre of 8-ring channels) while the nitrogen atom is located closer to the wall of the channel. Such an orientation of methylamine molecules suggests that N...H...O hydrogen bonds may play an important role in the formation of ACP-GIS2 framework. This also serves to explain why, under similar experimental conditions, the gismondine framework with $(\text{CH}_3)_4\text{N}^+$ is not found even though the aluminosilicate TMA gismondine structure is already known⁸.

Merlinoite ($1.5 \leq \text{Co/Al} \leq 3$). Similar to chabazite and gismondine analogues, different structures with the merlinoite topology have been prepared. These are, to our knowledge, the first non-aluminosilicate merlinoite analogues and also provide a synthetic route to the merlinoite framework using amines as structure-directing agents. The methylamine is likely to be the guest species in ACP-MER1 whereas in ACP-MER2, the ethylenediamine is statistically distributed at two orientations and NH_4^+ is likely to be the other charge-balancing extra-framework species. Several other amines including homopiperazine can serve as the source of ethylenediamine in the synthesis of ACP-MER2.

Phillipsite ($\text{Co/Al} \approx 3$). It has been demonstrated that 1,3-diaminopropane templates the formation of the thomsonite type structure. Using 2,2-dimethyl-1,3-propyldiamine, we have synthesized a phillipsite analogue. This shows that a relatively small change in the shape and charge volume ratio of the organic amines dramatically changes the framework topology from the thomsonite type to the phillipsite type. The framework topology of phillipsite has not been seen previously in the non-silicate system; neither has this topology previously been formed with organic amines. One synthetic form has been reported, but the structure characterization was based on the similarity of the powder pattern to that of natural phillipsites²⁸.

Our syntheses of gismondine, merlinoite and phillipsite analogues show that we are able to make framework structures with subtle differences by varying the charge and geometry of amines. Gismondine, merlinoite and phillipsite all contain 'double crankshaft' (cc) chains. They can also be considered as superimposed layers of 4.8.8 type nets. In each 4.8.8 sheet, one free corner of each tetrahedral atom can point up or down from the layer. For each 8-ring in

gismondine, four adjacent corners are directed upwards while the other four corners are directed downwards (UUUUDDDD). In phillipsite, six adjacent corners are up and the other two are down (UUUUUUDD). Merlinoite has double 8-rings in addition to the UUDUUDD configuration.

ACP-1 ($\text{Co/Al} \approx 8.0$). We have synthesized a framework topology that consists of a body-centred cubic arrangement of the double 4-ring units (D4R). Although this topology is simple, it has not previously been found in natural or synthetic compounds. But because this topology represents an ideal zeolite structure type, it has long been recognized and was once proposed as the structure type for zeolite Na P1 (ref. 30) and the mineral garronite¹. These last two materials have been subsequently identified as having the gismondine structure⁸. A diagram showing the body-centred cubic arrangement of the D4R units is shown in Fig. 2. Ethylenediamine molecules are located inside 8-ring channels and adopt two statistical orientations, similar to that in ACP-MER2. At the centre of each D4R unit, there is a water molecule, 2.31 Å away from four metal T-atom sites of the D4R unit. Similar to gismondine, phillipsite and merlinoite, ACP-1 can be considered as constructed from the 4.8.8 net when viewed down any of the three axial directions. Unlike merlinoite which has double 8-rings, the configuration of the free corners in each 8-ring is exclusively UUDUUDD in ACP-1. ACP-1 has three-dimensional 8-ring channels.

ACP-2 and GCP-2 ($\text{Co/T}^{3+} = 3$). Two new structures with the same topology have been prepared. ACP-2 and GCP-2 are isostructural and consist of double saw (ss) chains²⁹ propagating along the c -axis. The ss chains are joined through rows of tetrahedral atoms into a layer shown in Fig. 3. The three-dimensional framework, which has 4-, 6- and 8-rings, is built from the repeat of such a layer along the b -axis. These layers are held together through T–O–T linkages between the above-mentioned rows of tetrahedral atoms and there is no direct connection between the ss chains throughout the framework. Eight-ring channels along the c -axis are shown in Fig. 4. There are two different loop configurations of T-atoms (types A and C32)³¹. Either type is common in known zeolites, but the combination of them in the same structure has not been previously reported⁸. ACP-2 has two-dimensional 8-ring channels. Another new structure we synthesized, UCSB-4 (Table 1), also consists of 4-, 6-, and 8-rings and has the same combination of two different T-atom loop configurations as ACP-2 and GCP-2. However, the framework topology of UCSB-4 is different from that of ACP-2.

Conclusion

We have described a family of transition-metal-based zeolite-type structures. The methodology used in this work is based on the use of template charge and shape to define framework composition and topology. Framework-guest charge matching is achieved by varying both the organic charge/volume ratio and the framework charge by incorporation of mixed charge metal cations. The approach used in the syntheses of these transition-metal-based zeolite analogues can be applied to systems with diverse structural types and chemical compositions. It is possible to make not only zeolite analogues, but also structural analogues of other silicate structures. For example, we have synthesized two aluminum cobalt phosphates (ACP-FL1 and ACP-FL2, Table 1) with the feldspar structure. We have expanded the range of chemical compositions even for zeolite-type structures based on non-transition metals. Analogues of AlPO_4 -18 and faujasite (CAP-AEI1 and CAP-FAU1, Table 1) have been synthesized with a transition-metal concentration (in molar ratio) as high as 33% at metal-atom sites. For comparison, no more than 4% of Al sites have previously been substituted by cobalt in AlPO_4 -18 (ref. 32). The broad scope of the methodology suggests that many more zeolite analogues and novel framework structures are now accessible. These structures give the zeolite-type materials unique structural, chemical and other properties through the incorporation of transition-metal atoms in large concentrations, and could broaden the areas of applications for zeolite-type materials.

Methods

Synthesis. The typical synthesis conditions are given here using the synthesis procedures of ACP-THO1, ACP-SOD1 and ACP-1 as examples. The synthesis conditions for other structures are very similar to those given below, with relatively small variations in pH and the reactant composition. Gallium thomsonite analogues were prepared from gallium oxide obtained by dissolving gallium metal in nitric acid. AZP-THO1 was prepared using zinc oxide as the zinc source. Solution A mentioned below was prepared by mixing aluminum isopropoxide (23.41 g) with 85% H_3PO_4 (23.12 g) and ethylene glycol (101.00 g), followed by stirring at room temperature for ~24 h. ACP-THO1. Crystals were grown by first mixing $\text{CoCO}_3 \cdot x\text{H}_2\text{O}$ (0.64 g, Aldrich) with 85% H_3PO_4 (1.71 g, Fisher). After gentle stirring, ethylene glycol (11.29 g, Fisher) and aluminum isopropoxide (0.58 g, Fluka) were added. The mixture was stirred at room temperature until homogeneous, then 1,3-diaminopropane (1.03 g, Aldrich) was slowly added until the pH of the gel was 7.0. The gel was stirred at room temperature for 1 h, then transferred to a Teflon-coated steel autoclave, and heated at 180 °C for 4 d. Pure blue fibre-like crystals were recovered by standard vacuum filtration and drying techniques. ACP-SOD1. $\text{CoCO}_3 \cdot x\text{H}_2\text{O}$ (2.28 g), distilled water (17.68 g) and 85% H_3PO_4 (4.60 g) were stirred together in a 100 ml beaker for 10 min. Solution A (3.23 g) and piperazine hexahydrate (1.31 g, Fluka) were then added. The mixture was stirred until the piperazine was completely dissolved. A final pH of 7.9 was obtained with the addition of propylamine (2.48 g, Acros). The mixture was stirred at room temperature for 2 h, then transferred to a Teflon-coated steel autoclave and heated at 180 °C for 4 d. The product consisted of large, blue tetragonal prismatic crystals and pink powder and could be separated by sonication and decantation. ACP-SOD1 could also be prepared without the presence of propylamine, but the product then consisted of a mixture of different crystals and was more difficult to separate. ACP-1. $\text{CoCO}_3 \cdot x\text{H}_2\text{O}$ (0.77 g) was mixed with 85% H_3PO_4 (1.57 g), ethylene glycol (13.75 g) and solution A (1.98 g). Ethylenediamine (1.0 ml, Aldrich) was then added to adjust the pH of the gel to 8.4. The mixture was heated in a Teflon-coated steel autoclave at 180 °C for 4 d. Blue tetragonal bipyramidal crystals with the largest dimension up to 150 μm were recovered together with small, unidentified blue crystallites.

Elemental analysis. Quantitative elemental analyses were carried out on a Cameca SX-50 electron-probe microanalyser equipped with five wavelength-dispersive X-ray spectrometers. The beam damage observed for some samples might affect the accuracy of the results. In general, the observed values from the electron probe analyses agreed with X-ray diffraction derived results within

~1.0 wt%, but deviations >1.0 wt% (but usually <2.0 wt%) were also observed. The calculated values (in mass per cent, based on formula in Table 1) and experimental values in parenthesis are: ACP-ANA1: 3.88 (3.47) Al, 16.9 (18.6) Co and 13.4 (12.7) P; ACP-CHA4: 7.71 (7.74) Al, 16.8 (18.31) Co and 17.7 (17.0) P; ACP-GIS2: 8.76 (8.11) Al, 19.14 (17.91) Co and 20.12 (21.22) P; ACP-GIS3: 8.38 (7.93) Al, 18.3 (18.3) Co and 19.2 (18.6) P; ACP-SOD1: 5.21 (5.03) Al, 22.76 (20.70) Co and 17.94 (16.75) P; ACP-SOD2: 4.96 (4.07) Al, 21.67 (22.33) Co and 17.08 (16.91) P; ACP-2: 3.90 (4.05) Al, 25.55 (22.60) Co and 17.91 (16.93) P. Experimental values in mass percent and relative atom numbers in parenthesis are ACP-CHA1: 0.95 (1.38) Al, 27.91 (18.68) Co and 14.64 (18.64) P; ACP-GIS1: 2.64 (4.29) Al, 24.69 (18.39) Co and 16.74 (23.72) P; ACP-1: 1.53 (2.50) Al, 26.70 (19.99) Co and 15.86 (22.59) P; ACP-MER1: 5.38 (8.67) Al, 21.73 (16.03) Co and 17.36 (24.36) P.

Thermal analysis. The thermogravimetric analyses and differential thermal analyses were performed in static air with a heating rate of 5 °C min⁻¹ from 30 to 1,000 °C. Sample weights were 49–51 mg. For ACP-THO1 and ACP-THO2, the total observed weight losses between 340 to 640 °C are 20.5% and 20.0%, respectively (calculated, 20.7%). The thomsonite framework is not retained when amines are lost on heating. ACP-ANA1 has no weight loss and shows no obvious thermal events up to 1,000 °C. ACP-GIS2 has two separate weight losses (10.2% and 1.8%) starting at 400 and 860 °C, respectively, but the framework is lost at ~400 °C. Some other phases, including ACP-MER1 and ACP-CHA1, also show weight losses in two or more separate steps, but they do not retain their structures on calcination. Studies on the thermal stabilities of this family of materials are in progress.

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The crystal structure of the asymmetric GroEL–GroES–(ADP)₇ chaperonin complex

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Chaperonins assist protein folding with the consumption of ATP. They exist as multi-subunit protein assemblies comprising rings of subunits stacked back to back. In *Escherichia coli*, asymmetric intermediates of GroEL are formed with the co-chaperonin GroES and nucleotides bound only to one of the seven-subunit rings (the *cis* ring) and not to the opposing ring (the *trans* ring). The structure of the GroEL–GroES–(ADP)₇ complex reveals how large *en bloc* movements of the *cis* ring's intermediate and apical domains enable bound GroES to stabilize a folding chamber with ADP confined to the *cis* ring. Elevation and twist of the apical domains double the volume of the central cavity and bury hydrophobic peptide-binding residues in the interface with GroES, as well as between GroEL subunits, leaving a hydrophilic cavity lining that is conducive to protein folding. An inward tilt of the *cis* equatorial domain causes an outward tilt in the *trans* ring that opposes the binding of a second GroES. When combined with new functional results, this negative allosteric mechanism suggests a model for an ATP-driven folding cycle that requires a double toroid.

Chaperonins were initially identified in eubacteria, and in the evolutionarily related mitochondria and chloroplasts, but a second family was subsequently identified in archaea and in the eukaryotic cytosol (for review see refs 1–3). Chaperonins seem to provide kinetic assistance to the process of folding newly translated or newly translocated polypeptides. Although steric information specifying the final native form of a protein is provided by its primary sequence⁴, there is an apparent disposition under certain conditions *in vivo* for many polypeptides to misfold and aggregate irreversibly. By preventing this from happening^{5–7}, chaperonins seem to facilitate production of the native state under conditions in which the native form would otherwise not be achieved. Overall, their actions increase the yield of properly folded proteins but rarely increase the inherent rate of the folding reaction.

The bacterial chaperonin GroEL and its co-chaperonin GroES, the best-studied of these chaperonins, are coexpressed from a common operon (*GroE*) in *Escherichia coli*. GroEL (L for large) contains 14 identical subunits of relative molecular mass 58,000 (*M_r* 58K) that are assembled as two heptameric rings stacked back to back. GroES (S for small) contains seven identical 10K subunits assembled as one heptameric ring. Both chaperonins are essential in protein folding under all cell conditions, as demonstrated by mutational analysis^{8–10}.

GroEL is thought to promote productive folding through two actions. One is to bind a non-native (but rarely native) polypeptide to the walls of the GroEL central channel, mainly through hydrophobic contacts^{11–15}. This serves to diminish aggregation with other non-native polypeptides and to partly unfold kinetically trapped

intermediates, allowing them another chance at productive folding^{16–19}. The second action is to facilitate folding after the polypeptide is released into an expanded and shielded central channel^{20–22}. The two assisting actions of GroEL are associated with distinct, and definable, conformational states of GroEL–GroES^{23,24}, interconverted by the action of ATP, which is cooperatively bound and hydrolysed within one ring^{25–28}. It has been established that a folding-active state of GroEL is an asymmetric complex with GroES bound at one end in the presence of ATP^{20–22}. Depending on the polypeptide, successful folding can require one or many rounds of binding and release^{16,20,29–35}.

Our current understanding of the action of GroEL recognizes the importance of the timing and synchronization displayed by this highly allosteric system, which coordinates the binding and hydrolysis of ATP, the binding of GroES, and the binding and release of polypeptide. The architecture of GroEL, the GroEL–GroES complex and its polypeptide and nucleotide complexes were first described at low resolution (30 Å or lower) by negative-stain and cryo-electron microscopy^{23,36–39}. The crystal structures of GroEL and GroES have been determined as separate assemblies to atomic resolution^{11,40}, as has the crystal structure of GroEL fully complexed with 14 molecules of a non-hydrolysable ATP analogue, ATP-γS²⁸, and more recently the crystal structure of an isolated apical domain of the GroEL subunit⁴¹. Although these crystal structures have provided a valuable initial stereochemical framework for biochemical and mutational studies, the highly allosteric mechanisms by which the GroEL, GroES, nucleotides and polypeptides function in an integrated cyclic fashion requires high-resolution structural

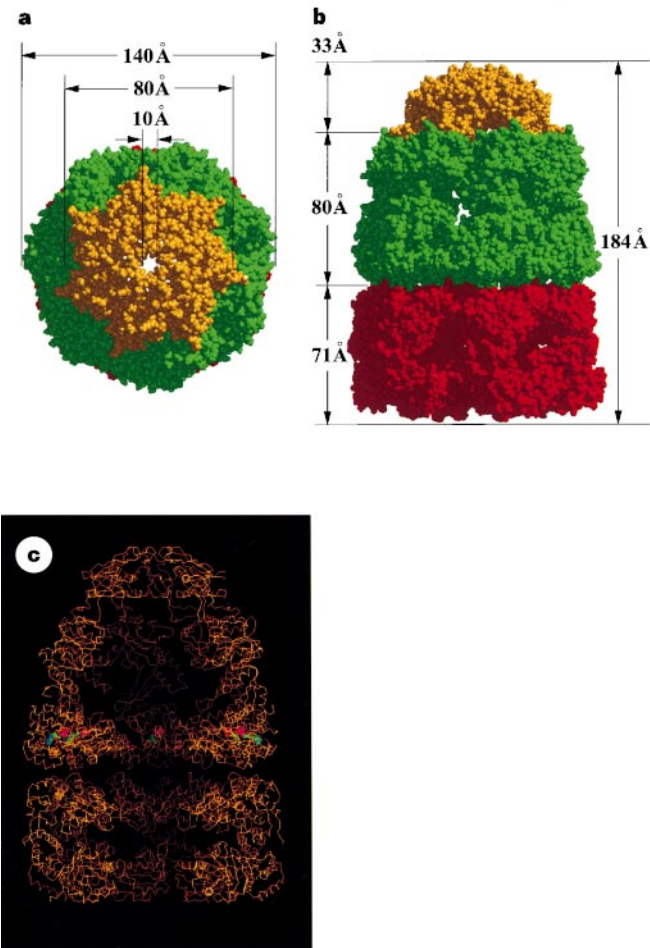


Figure 1 Overall architecture and dimensions of the GroEL-GroES complex. **a**, Van der Waals space-filling model of the entire complex in a top view looking down from the GroES-binding (*cis*) side; **b**, as **a**, but in a side view. The complex is colour coded as follows: *trans* GroEL ring, red; *cis* GroEL ring, green; GroES, gold. **c**, α drawing of the 'inside' of the GroEL-GroES complex. The view was produced by cutting the assembly open with a plane containing the 7-fold axis. ADP molecules bound to *cis* GroEL ring are shown as space-filling models. **a**, **b**, Produced using MidasPlus (Computer Graphics Laboratory, University of California, San Francisco); **c**, produced using program O⁵³.

studies of the appropriate complexes.

We have used X-ray crystallography at 3.0 Å resolution to determine the three-dimensional structure of a complex formed by wild-type GroEL, GroES and seven bound ADP molecules. The dramatic change in the overall structure of the GroEL-GroES complex, as compared with unliganded GroEL, can be attributed to the *en bloc* domain movements in the subunits of the ring to which GroES is bound (the *cis* ring). The rearrangements more than double the volume of the cavity, increase the hydrophilicity of the cavity's lining, and suggest a mechanism for peptide release. These movements also cause significant changes in the nucleotide-binding pocket, which accounts for the highly asymmetrical binding of ADP to only the seven *cis* subunits and provides plausible stereochemical links between the binding of GroES and the binding of ATP. When coupled to the preservation of the interface between rings, these architectural changes may also underlie the negative allosteric behaviour between rings³⁰ and provide a molecular mechanism for the action of ATP and the double toroid in the chaperonin-assisted folding cycle.

Structure determination

We chose to crystallize GroEL-GroES complexed with ADP because it is the most accessible intermediate in the reaction cycle. Full-length wild-type *E. coli* GroEL and GroES were overexpressed and purified separately. The GroEL-GroES complex was formed in a high concentration of ADP (25 mM), but was purified and crystallized in a lower concentration (50 μM) to ensure that only high-affinity sites would be occupied. Microseeding was used to grow large, single crystals reproducibly. These crystals usually diffracted X-rays weakly and anisotropically to an average resolution of 3.0 Å (Table 1). The crystals are of space group *P*2₁2₁2 with one GroEL molecule (14 subunits), one GroES molecule (7 subunits) and 7 ADP molecules in the crystallographic asymmetric unit.

The structure of GroEL-GroES complexed with 7 ADP molecules was determined by using a combination of molecular replacement and non-crystallographic molecular averaging (Table 1). Refinement of this structure resulted in an *R*-value of 24.8% and a free *R*-value of 29.1% at 3.0-Å resolution (Table 1). The refined model includes all residues except the carboxy-terminal 23 residues of GroEL which are not visible, as was the case in the crystal structures of unliganded¹¹ and ATP-γS-bound GroEL²⁸.

Overall architecture

The overall structure and dimensions are shown in Fig. 1. The two GroEL rings (*cis* and *trans*) stack back-to-back, forming a channel in

Table 1 Structure determination and refinement

Rotation search		Euler angles			Highest peak			Highest false peak	
Search structure		θ_1	θ_2	θ_3					
GroEL 14-mer		5.8	1.5	5.8	12.5 σ			7.9 σ	
Translation search		Fractional coordinates			Highest peak			Highest false peak	
		x	y	z					
		0.36	0.16	0.02	25.6 σ			15.5 σ	
Structure refinement									
Resolution limits (Å)	6.00	4.76	4.16	3.78	3.51	3.30	3.14	3.00	Total
No. of reflections	31,561	30,851	30,450	30,491	30,459	30,431	30,124	28,317	242,684
Completeness (%)	97.8	97.9	97.1	97.6	97.7	97.7	96.8	91.2	96.7
$\langle I \rangle / \sigma$	19.7	14.3	13.3	9.4	5.9	3.8	2.6	1.8	9.1
R_{sym}	0.059	0.091	0.101	0.138	0.202	0.278	0.394	0.530	0.121
R -factor (data > 2.0 σ)	0.196	0.241	0.231	0.251	0.271	0.294	0.315	0.356	0.248
Free R -factor (data > 2.0 σ)	0.245	0.295	0.264	0.296	0.303	0.343	0.353	0.389	0.291
R.m.s. deviations	Bond lengths		0.010 Å		Bond angles		1.40°		

* $R_{sym} = \sum_i \langle |I_i - \bar{I}_i| \rangle / \sum_i I_i$, where $\langle |I_i - \bar{I}_i| \rangle$ is the average of the absolute deviation of a reflection I_i from the average $\bar{I}_i$ of its symmetry and Friedel equivalents.

the centre of the rings, as in the unliganded GroEL structure (Fig. 1c)¹¹. Large *en bloc* movements of the apical and intermediate domains in the *cis* ring (Fig. 2) widen and elongate the *cis* channel. The GroES ring, assembled as in its stand-alone structure⁴⁰, caps the apical surface of the *cis* ring, anchoring the elevated orientation of the apical domains and closing off the end of the channel. The net result is a dome-shaped chamber that has the elevated apical domains as its walls and the GroES cap as its roof. The three rings share one nearly exact 7-fold rotational axis. The smoothness of the union between the GroEL *cis* ring and the cap results in a 'bullet'-shaped complex^{36,37}. The equatorial domains in the *cis* ring undergo little change except for small but potentially important local shifts in the nucleotide-binding pocket and a slight (4 deg) *en bloc* 'inward' tilt that alters the orientation but not the local details of the interface to the *trans* ring. In contrast to the dramatic structural changes in the *cis* ring, the *trans* ring closely resembles that of unliganded GroEL.

Domain shifts in the *cis* ring

The structures of the GroEL subunits in the *cis* and *trans* rings are shown in Fig. 2d, e. Whereas the structure of the *trans* GroEL subunit deviates only slightly from that of the unliganded GroEL (root mean square (r.m.s.) deviation of 1.60 Å for C α atoms; Fig. 2b, e), the structure of the *cis* GroEL subunit shows profound differences, which are attributed to dramatic domain rearrangements involving both the intermediate and apical domains (Fig. 2b, d). First, the intermediate domain swings down towards the equatorial domain and the central channel, pivoting approximately 25 deg around Pro 137 and Gly 410, which form a slender link to the equatorial domain (Fig. 2c). The movement closes the nucleotide-binding site that is located on the top inner surface of the equatorial domain and generates numerous new interactions with the equatorial domain, both within the same subunit and with a neighbouring subunit. These interactions seem to sterically impede the dissociation of ADP from the *cis* ring, and they structurally relate GroES binding to the presence of ATP and to ATP hydrolysis in the *cis* ring⁴². Second, the apical domain swings up 60 deg relative to the equator and twists around the 'long axis' of the domain by about 90 deg, leading to an interaction with the 'mobile loop' of GroES (see below). The pivot point of the apical domain's movement is again a slender link, in this case a pair of glycine residues (Gly 192 and Gly 375) between the intermediate and apical domains (Fig. 2c). The position of the apical domain is linked through this hinge to the nucleotide-induced/stabilized movement of the intermediate domain. The movement of the hinge in response to nucleotide directly couples the binding of GroES to the presence of the nucleotide (and vice versa). Both intermediate and apical domain movements are largely *en bloc*; the r.m.s. deviations for superimposition of the intermediate and apical domains of the *cis* subunit upon their unliganded counterparts are 0.91 and 1.66 Å, respectively. The domain movements, especially those of the apical domains, dramatically reshape the cavity formed by the *cis* GroEL ring and GroES, doubling the volume of the cavity (consistent with an electron-microscopy study²³) and changing the polypeptide binding properties of the cavity lining (see below).

GroES

Each GroES subunit is folded into a single domain, which contains nine β -strands with one exceptionally long loop between strand 2 and 3 (Fig. 2f). The GroES heptamer ring in the complex is very similar to that in the stand-alone structure except for residues Glu 16 to Ala 32 in the long loop, which were visualized in only one of the seven GroES subunits in the stand-alone structure but are seen in all seven of the GroES subunits of the complex. In a proton nuclear magnetic resonance study⁴³ these residues appeared highly mobile in uncomplexed GroES (hence the term 'mobile loop') but become more structured upon interacting with GroEL, and there-

fore were thought to form the interface between GroEL and GroES. The electron density in the averaged map was good enough to determine structures for all seven GroES 'mobile loops' in the complex and, as predicted, they do form the GroEL–GroES interface. As anticipated⁴⁰, the loop's conformation in the complex differs from its one representation in the GroES stand-alone crystal structure. The long mobile loop is a β -hairpin (Fig. 2f), as indicated by a nuclear Overhauser enhancement (NOE) transfer experiment⁴⁴, which swings down (9 Å) and outwards (20 Å) from the β -core to interact with GroEL. The GroEL–GroES interface consists of mostly aliphatic side chains, including Ile 25, Val 26 and Leu 27 of GroES and Leu 234, Leu 237 and Val 264 of GroEL. The GroEL residues cluster on a pair of helices (H and I), and the study of the unliganded GroEL structure^{11,12} suggests they are involved in substrate polypeptide binding. The structure is consistent with mutational changes in the mobile loop that disrupt GroES binding, although it does not suggest an obvious mechanism by which mutations in the hinge regions of GroEL (V174F, V190I and G375S) suppress the mutagenic disruption of GroES binding⁴⁵.

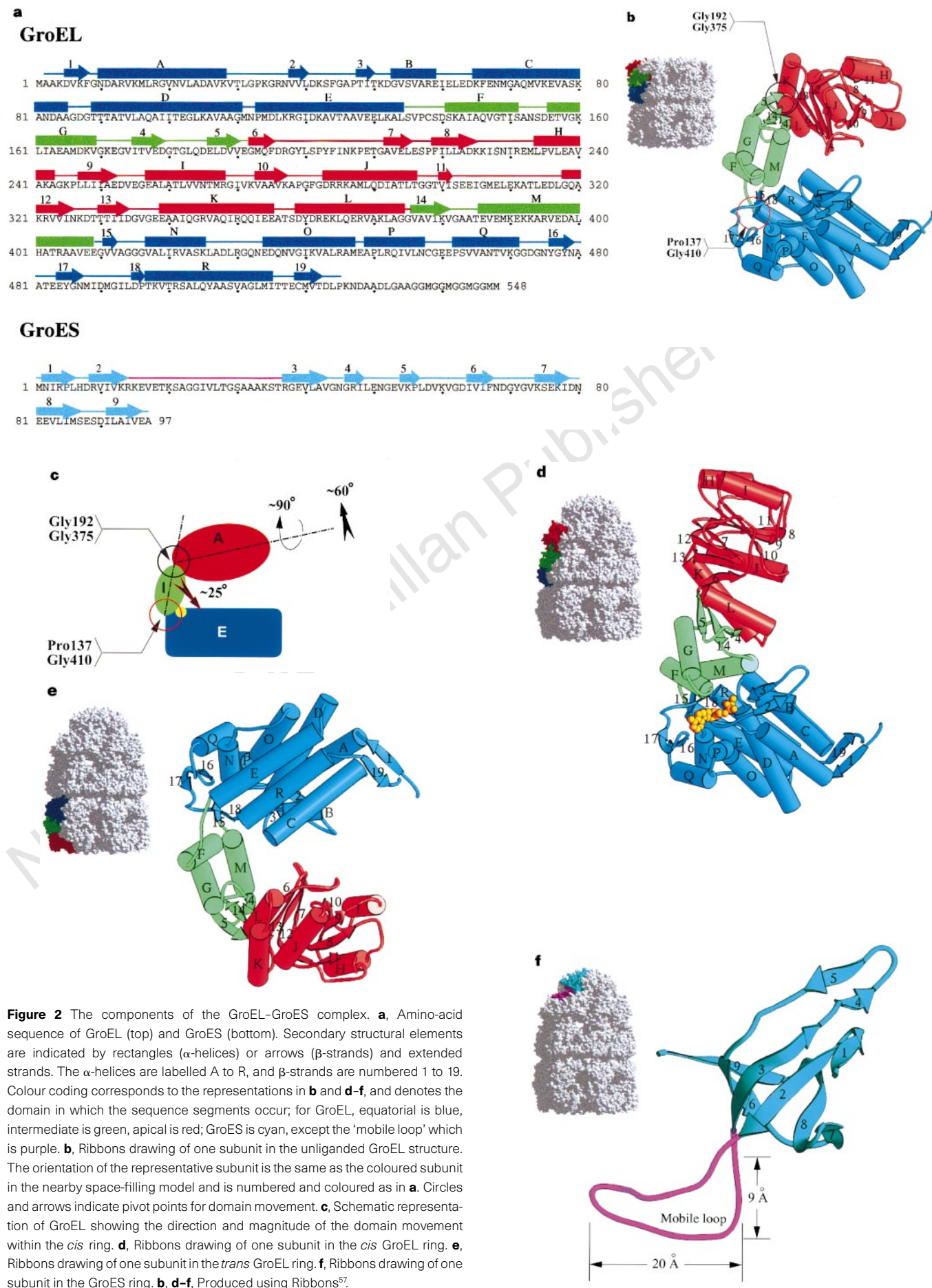
The nucleotide-binding site

The nucleotide-binding pocket is on the top surface of the equatorial domain facing towards the central cavity²⁸. The 7-fold molecular averaged map showed well-defined electron density for the ADP molecule and a metal ion (refined as magnesium) in the *cis* ring nucleotide-binding site (Fig. 3a). In contrast, nucleotide-binding sites in the *trans* ring are empty. The structure of GroEL–GroES–(ADP)₇ was refined with all seven *cis* sites fully occupied and the seven *trans* sites empty. A simulated-annealing omit map—in which ADP, a magnesium ion and adjacent amino acids were deleted from the model before the refinement—showed well-defined electron density for the omitted components; these being ADP, magnesium ion and the adjacent protein in the *cis* GroEL ring.

The nucleotide-binding site in the fully saturated GroEL–ATP γ S structure is largely open²⁸, so nucleotide can enter and exit the binding site without steric obstruction. Upon GroES binding, residues of the helices F and M of the intermediate domain clamp onto the equatorial domain and close the nucleotide-binding site. Ile 150 of helix F forms a van der Waals interaction with the sugar moiety of the ADP, and helix M contributes the carboxylate oxygen of Asp 398 to the Mg²⁺ ion coordination cage (Fig. 3b, c). Asp 398 is required for ATP hydrolysis and progression of the chaperonin cycle⁴², which has been shown⁴⁶ to depend on GroEL domain rearrangements. Except for the absence of a second metal ion-mediated interaction with the α -phosphate, the specific interaction of ADP with the equatorial domain largely mirrors that reported in the GroEL–ATP γ S structure²⁸.

New interfaces in the *cis* ring

In the GroEL–ATP γ S complex, the bound ATP- γ S (in the absence of GroES) caused only two noticeable structural changes when compared with the unliganded GroEL structure²⁸. One was a significant axial translation of helix C on the 'top' of the equatorial domain. The second was a movement of the stem loop (Lys 34 to Asp 52) whose antiparallel stem forms an essential parallel β -contact with the neighbouring subunit through a β -strand near the C terminus (strand 19). This connecting strand precedes the disordered 23 C-terminal residues projecting into the floor of the central cavity. The movement of the stem loop was ascribed to an adjacent metal ion-mediated contact between the α -phosphate and backbone carbonyls at the base of the stem, which shifts the loop position by as much as 4 Å. The equatorial domain of the *cis* ring in the GroEL–GroES complex shows the same nucleotide-induced shifts in helix C and the Lys 34–Asp 52 stem loop. More importantly, the now radically reoriented intermediate domain makes contact with these same two nucleotide-shifted substructures, one within the same subunit (the Lys 34–Asp 52 stem loop), the other in



the neighbouring subunit (helix C). Residues from helix M of the reoriented intermediate domain of the *cis* ring form contacts with the loop of the stem loop within the same subunit and with helix C of the neighbouring subunit (Fig. 4). In addition, the loop of the stem loop forms a more extensive interaction with a neighbouring helix C. This establishes a structural connection within the *cis* ring that coordinates the binding of nucleotide and GroES.

It has been widely observed that the binding of GroES to GroEL is dependent on adenine nucleotide⁴⁷. The nucleotide-induced shift of the two substructures could well be a prelude to GroES binding. Once GroES binds, the domain shifts in the *cis* ring block the nucleotide's entry and exit, and stabilize the shifts of the substructures induced by nucleotide binding. Thus the consequences of GroES binding are coupled to the structural transitions caused by ATP binding both directly (blocking the site) and indirectly (stabilizing the substructure shifts). The fact that the binding of GroES and nucleotide each causes mutually supportive interactions explains the functional interplay between GroES and nucleotide.

The central cavity

The central channel of GroEL functions as two cavities, one in each ring, that are separated from each other by the crystallographically disordered 23-amino-acid C-terminal segments of the seven subunits. In electron micrographs, these segments appear to coalesce and block the central channel at the level of the equatorial domain³⁹, which is consistent with low-angle neutron-scattering

experiments⁴⁸. This is apparently functional, as a polypeptide cannot escape through the equatorial segments of a single ring mutant²². This entry and exit of polypeptide seem to be restricted to the apical end of each ring. Presumably the surface presented to the channel by a single ring of apical subunits is sufficient to entrap a non-native polypeptide (that is, the *trans* side cavity of the GroEL–GroES complex is probably the acceptor state for polypeptide *in vivo*). As in the unliganded GroEL structure, the *trans* cavity is cylinder shaped and rather uniform in diameter, except at the intermediate level where irregular outward bulges occur (Fig. 5c). The total volume of that cavity (excluding the bulges) is measured as 85,000 Å³. If the partial specific volume of a folded native protein is 1.23 Å³ per Da, and if passage of peptide through the equatorial level is prohibited, then there is just enough space for a native protein of relative molecular mass 70,000 (*M_r* 70K) assuming a perfect fit, but a more loosely packed, non-native polypeptide that could fit completely inside the cavity would have to be much smaller. Of course, in the absence of GroES, portions of bound polypeptide can protrude from the central channel, as demonstrated by electron microscopy and small-angle neutron scattering^{39,48}. On the *cis* side, the reorganized GroEL domains stabilized by the binding of nucleotide and the GroES cap form a dome-shaped, smooth-walled cavity, about 80 Å in diameter and 85 Å high (Fig. 5c). This cavity has a volume of about 175,000 Å³, more than double the original size and easily capable of accommodating a globular protein or even an expanded-volume molten globule intermediate

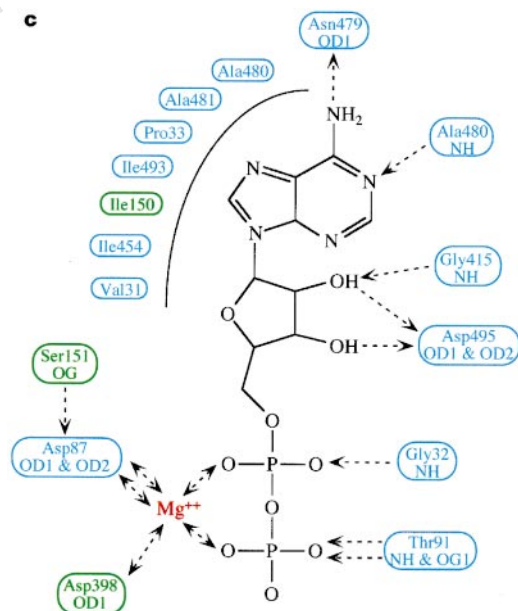
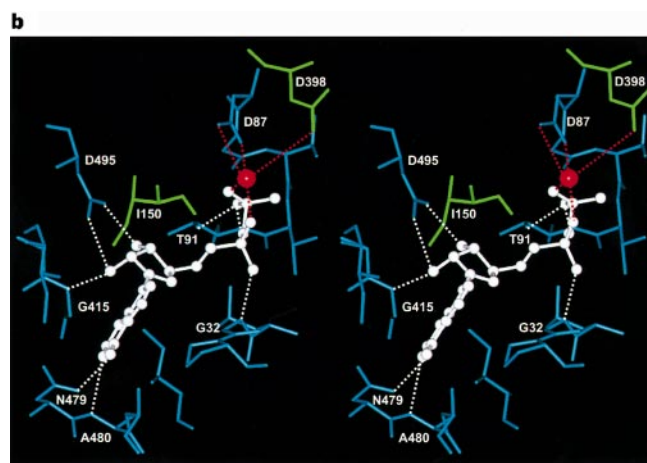
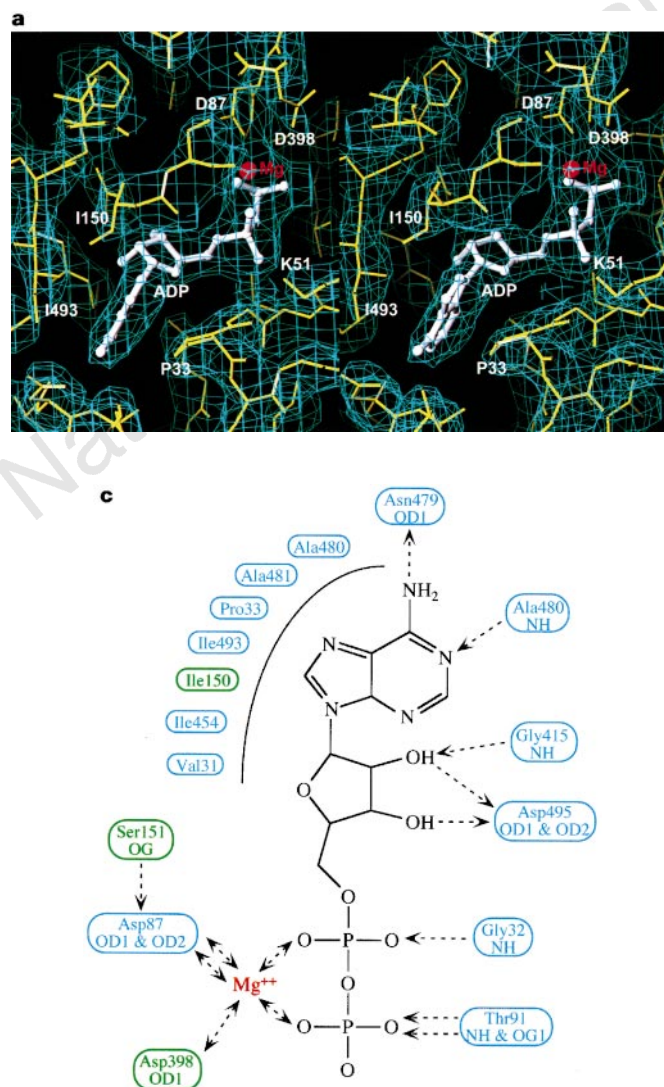


Figure 3 Nucleotide-binding site in the *cis* ring of the GroEL–GroES complex. **a**, Stereo pair of a SigmaA-weighted $2F_o - F_c$ electron-density map contoured at 2σ showing the ADP-binding pocket in a subunit of the *cis* GroEL ring. ADP, white, protein, yellow. 'Mg' denotes a bound magnesium ion. **b**, Stereo view of direct Mg^{2+} –ADP interactions with the protein. The protein is shown as a skeletal model and is coloured as in Fig. 2. The ADP is a white ball-and-stick model, the Mg^{2+} is a red sphere, hydrogen bonds are shown as white dotted lines and magnesium coordinations are red dotted lines. **c**, Schematic representation of direct Mg^{2+} –ADP interactions with the protein (less than 3.2 Å). Amino-acid residues from the equatorial domain are blue, and those from the intermediate domain are green, as in Fig. 2. Hydrogen bonds are shown as single-arrow dashed lines, and magnesium coordinations are shown as double-arrow dashed lines. Residues interacting with ADP through van der Waals contacts are shown along a curved line. OG, OG1, OD1, OD2 and NH stand for O_γ , $O_{\gamma 1}$, $O_{\delta 1}$, $O_{\delta 2}$ and peptide NH, respectively. **a**, Produced using O⁵³; **b**, produced using InsightII (BioSyr Technology).

of $M_r > 70K$.

Structure-based mutagenesis has identified nine residues that are required for binding non-native polypeptide on the surface of the apical domains facing the central cavity (of a *trans* ring)¹². These residues are clustered on three secondary structural elements: (1) a long loop between strands 6 and 7 (Tyr 199, Ser 201, Tyr 203 and Phe 204); (2) helix H (Leu 234 and Leu 237); and (3) helix I (Leu 259, Val 263 and Val 264) (Fig. 5a). In the GroEL–GroES complex, helices H and I have moved to the top of the subunit to form part of the GroEL–GroES interface, and the loop between strand 6 and strand 7 is elevated and rotated into the *cis* ring's newly formed interface between apical domains (Fig. 5b; also Fig. 4a, b). Thus the binding of GroES and nucleotide deprives the substrate polypeptide of its binding elements because the residues in the *cis* ring implicated in binding non-native polypeptide are now involved in either binding GroES directly (helices H and I) or supporting GroES binding indirectly by stabilizing the interface between elevated and rotated apical domains.

The surface of the expanded *cis* cavity now presents mostly polar residues. A sample of side chains that point into the cavity in the *cis* ring includes Asp 224, Lys 226, Ser 228, Glu 252, Asp 253, Glu 255, Glu 304, Lys 327, Asp 328, Asp 359 and Glu 363. Hydrophobic residues that bound the non-native polypeptide (presumably through hydrophobic interaction) in the cavity of the *trans* ring

are now used to stabilize interfaces that support the GroES complex, and have been replaced on the cavity walls by mostly polar residues (Fig. 5c, compare *cis* and *trans*). This switch in the chemical character of the cavity lining triggers dissociation of the non-native polypeptide from the wall of the cavity. The released polypeptide is now free to re-initiate folding as an isolated molecule in a much-enlarged cavity that has a hydrophilic lining conducive to burial of the substrate polypeptide's hydrophobic residues as it initiates folding into a native structure.

Allosteric communication to the *trans* ring

Superimposition of seven equatorial domains of the *cis* GroEL ring on those of the unliganded GroEL shows that the plane of the ring is slightly deformed upon GroES binding. In the *cis* GroEL ring, each subunit tilts about 4 deg towards the cylinder axis so that the inside of the ring is 3 Å lower than the original plane and the outside is 5 Å higher. Some of the largest shifts were observed for residues that are involved in cross-ring interactions: for example, the C α of Glu 434 moves 4.9 Å and the C α of Ala 109 moves 3.8 Å away from equatorial plane. Despite these shifts, the chemical details of the interface are maintained. To preserve the inter-ring contacts, the *trans* ring must shift in a complementary direction. This causes each *trans* subunit to tilt in the opposite direction, away from the central axis by about 2 deg. The scheme shown in Fig. 6 suggests that a

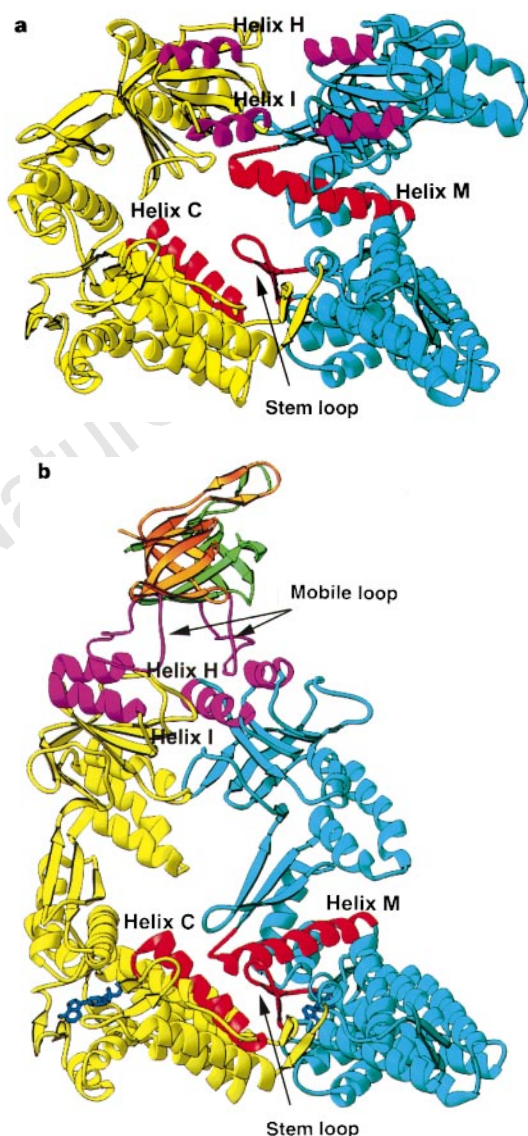
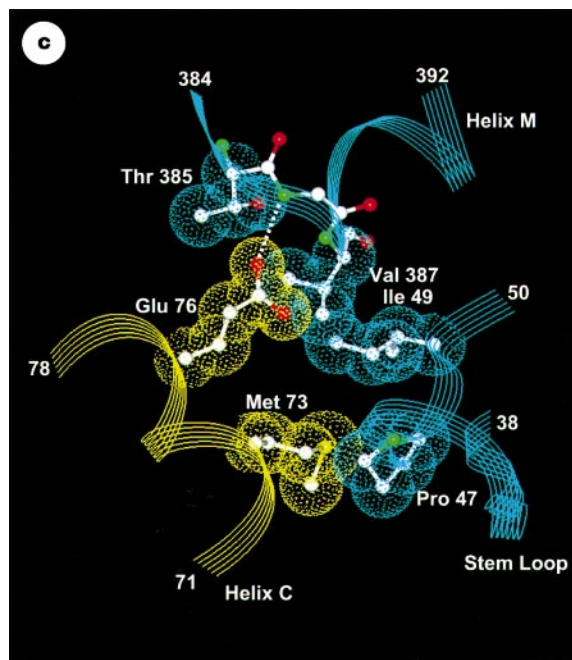


Figure 4 Inter-subunit contacts in the *cis* ring of the GroEL–GroES complex. **a, b**, Ribbons drawings for two adjacent subunits in *trans* (**a**) and *cis* (**b**) GroEL rings viewed from the inside of the ring. In each panel, the left GroEL subunit is yellow and the right subunit is cyan. Two GroES subunits (orange and green) are shown in **b**, along with two bound ADP molecules (blue). The substructures forming the new interface between equatorial subunits are red, and the substructures forming the GroEL–GroES interface are magenta. **c**, The site of contact at the new interface between equatorial subunits in the *cis* ring. The orientation is roughly the same as that of **b**. Side chains of residues at the contact surface are shown as ball-and-stick models: carbon, white; nitrogen, green; oxygen, red; sulphur, yellow. Van der Waals surfaces are represented by dot drawing. Shortened radii of 1 Å are used for clarity. Backbone traces are represented by a coiled-line ribbon drawing. Both the ribbon and surface are yellow for the left subunit and cyan for the right subunit. Hydrogen bonds are shown as white broken lines.



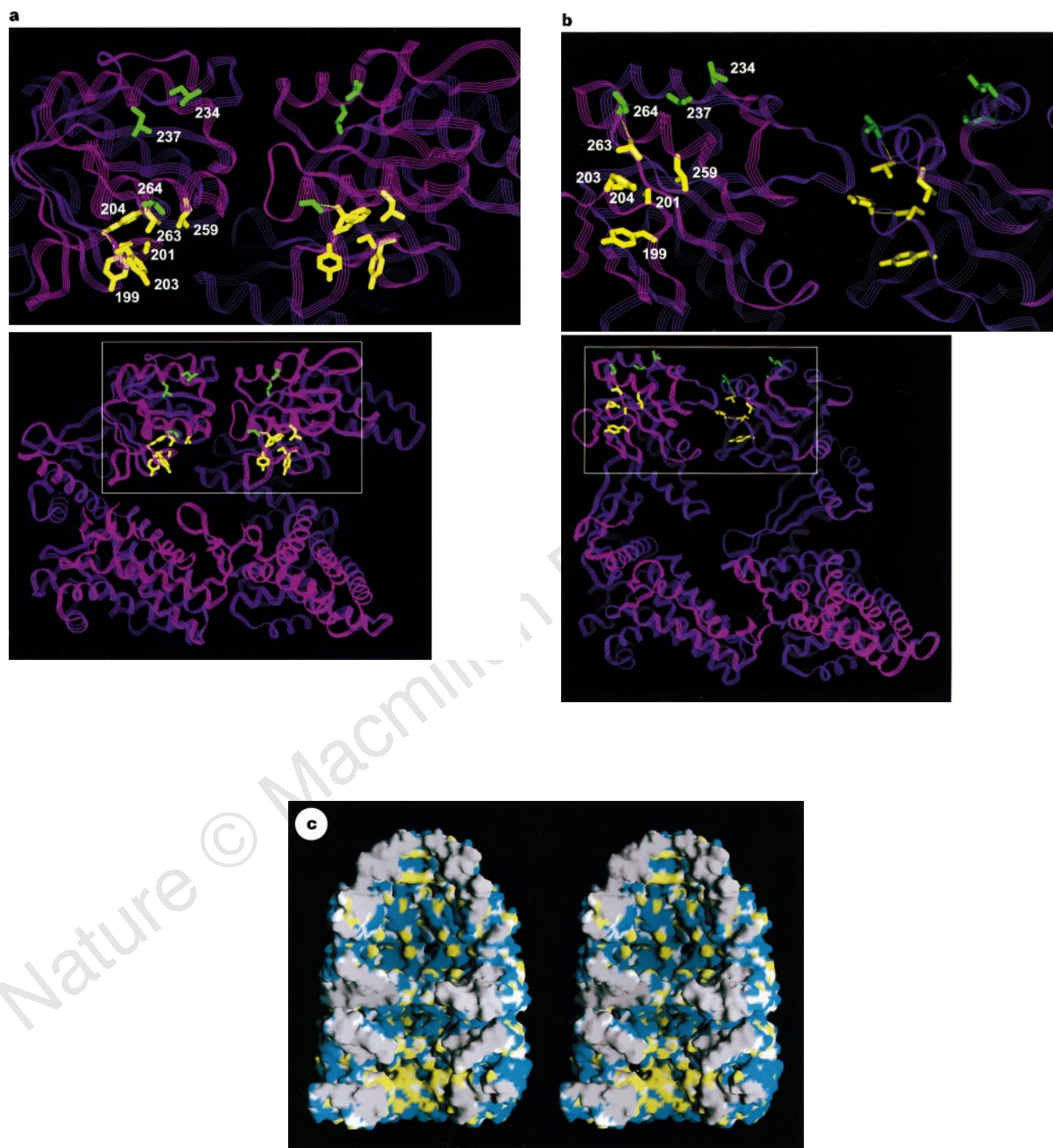


Figure 5 The change in the central cavity. **a**, Bottom, coiled-line ribbon drawing of two neighbouring subunits of the *trans* ring viewed from the central cavity; top, a close-up view of the rectangular area. Skeletal side chains denote residues involved in polypeptide binding as derived by mutagenesis¹². These residues, with the exception of Ser201, have hydrophobic side chains. **b**, Coiled-line ribbon drawing of two neighbouring subunits of the *cis* ring viewed from the central cavity in the same orientation and highlighted as in **a**. Note that these residues are moved away from the cavity surface. Three residues now form the GroES interface (green; Leu234, Leu237 and Val264), and the rest form the new apical GroEL subunit interface (yellow; Tyr199, Ser201, Tyr203, Phe204, Leu259 and Val263). **c**, Stereo pair of the accessible surface of the central cavity of the GroEL-

GroES complex. The complex is in the same orientation as in Fig. 1b. The three subunits from each of the rings nearest the viewer were removed to show the inside of the assembly. The solvent-exposed surface (assuming the assembly is complete) is coloured based on the underlying atoms: all backbone atoms, white; all hydrophobic side-chain atoms (Ala, Val, Leu, Ile, Met, Phe, Pro and Tyr), yellow; all polar and charged side-chain atoms (Ser, Thr, His, Cys, Asn, Gln, Lys, Arg, Asp and Glu), blue. All solvent-excluded surface at the subunit interfaces are grey. Note the yellow hydrophobic patches on the surface of the *trans* GroEL cavity and blue polar patches on the surface of *cis* GroEL cavity. **a**, **b**, Produced using InsightII (BioSym Technology); **c**, produced using Grasp⁵⁸.

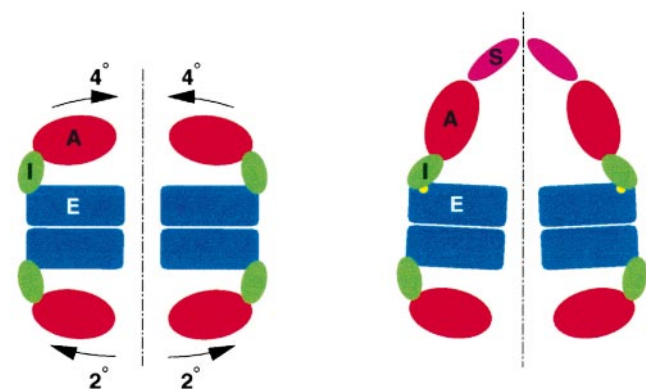


Figure 6 Schematic (exaggerated) representation of the *en bloc* tilt of the equatorial domains and the subsequent deformation of the equatorial plane. E, equatorial domain; I, intermediate domain; A, apical domain; S, GroES subunit. The inward tilt in the *cis* ring is 4° and the complementary outward tilt in the *trans* ring is 2°. The interface between *cis* and *trans* rings is smooth and conserved in detail irrespective of the presence or absence of the *cis* assembly.

symmetrical complex with GroES on both sides would strain the complementary interface. To preserve the interface, binding events in the *cis* ring oppose similar events in the *trans* ring. Thus the transmission of negative allosteric effects across the equatorial plane, like the positive effects within a ring, is primarily through *en bloc* movements rather than through conformational shifts within the domains. Unlike the mechanisms in most other allosteric systems, this expression of negative allostery depends on the preservation, rather than the alteration, of the interfacial contacts across the equatorial plane.

The molecular mechanism proposed above offers an explanation for both functional results⁴² and for the workings of the double toroidal architecture in the folding cycle. Binding of ATP, but not ADP or AMP-PNP, will release the most tightly bound non-native polypeptides into the domed folding cavity of the *cis* ring⁴², allowing them to initiate and, if sufficient time elapses, complete folding to a native state. Moreover, bound ATP, but not ADP or AMP-PNP, causes an ATPase-deficient assembly (mutant D398A) to maintain its structural integrity when challenged by low temperature and 0.4 M guanidium HCl. Most importantly, rings of the D398A mutant maintain their domed *cis* assembly and will not release nucleotide, GroES and folded polypeptide when the *trans* ring is exposed to ATP and GroES, as would be the case if bound ATP in the *cis* ring were hydrolysed to ADP⁴². The conclusion drawn from these observations, which is totally consistent with the role of nucleotides driving other molecular mechanical systems⁴⁹, is that the γ -phosphate of the nucleoside triphosphate contributes additional strong contacts (not observed in this structure) that stabilize the high-energy conformation, which in turn causes a change in the quaternary structure. Here, that change is the formation of a domed *cis* assembly. When the nucleotide loses its γ -phosphate, the triphosphate-stabilized structures are weakened and, under appropriate stress, the nucleoside diphosphate-bound structures relax back to the ground state, here the unliganded *trans* form. For the stereo-mechanical reasons described above, the opposite rings compete for the stability of a domed folding chamber in a system where ATP provides significantly greater stability than ADP to the assembly⁴². Thus the *cis* ring association of GroES is weakened by hydrolysis of ATP and becomes susceptible to disassembly (and release of ADP, GroES and polypeptide) when subjected to the outward 'tilting' stress imposed by the binding of ATP on the opposite ring. This explains the inability of the *cis* assembly on a single ring mutant to disgorge its ligand²², and shows why the GroEL-assisted dynamic folding cycle requires a double-toroid structure.

Conclusion

The structure of GroEL–GroES–(ADP)₇ provides information about several important features of the bacterial chaperonin-assisted protein-folding cycle. First, the stability of this asymmetric ADP-bound intermediate emphasized in early biochemical studies³⁰ is derived in part from the closing of the nucleotide-binding pocket

in the *cis* ring. The dramatic GroES-stabilized rearrangement of the intermediate domain that causes the steric block to ADP release is supported by the formation of new interfaces in the *cis* ring with substructures that have shifted in response to nucleotide binding. Thus GroES and nucleotide mutually support the maintenance of the complex. The intra-ring positive cooperativity of nucleotide binding is secured by GroES because GroES is a preassembled allosteric effector with a 'valence' of seven that can interact with all seven GroEL subunits of the *cis* ring at one time.

Second, the structure shows the mechanism by which peptide is released into the dome-shaped cavity. The apical domains are raised in a hinge-like manner and twisted, so that the nine mutationally identified residues responsible for peptide binding are removed from the channel surface and incorporated either into the interface with GroES or the interface between apical domains. This may explain why residues required for polypeptide binding are also required for GroES binding¹². Indeed, three of these residues (Leu 234, Leu 237 and Val 264) interact with GroES directly. The remaining six residues (Tyr 199, Ser 201, Tyr 203, Phe 204, Leu 259 and Val 263), however, stabilize the rearrangement needed to support GroES binding. The net effect is a large folding chamber, the lining of which is devoid of the hydrophobic residues that bind non-native polypeptide. In their places are polar residues, making the lining of the chamber a highly polar surface that will promote release of non-native polypeptide and favour commitment to the native state.

Finally, the behaviour of the two rings in the folding cycle reflects a strong negative cooperativity. Our results attribute the negative allostery to the *en bloc* inward tilt of the equatorial domains in the *cis* ring which, when coupled to the preservation of the interface between equatorial domains of opposing rings, requires a complementary outward tilt of the equatorial domains in the *trans* ring. This outward tilt opposes GroES binding. Recent results⁴² indicate that the binding of ATP stabilizes the *cis* assembly more firmly than the binding of ADP (or the conformationally distorted AMP-PNP). Thus binding of ATP and GroES to one ring will oppose similar events in the opposite ring until ATP is hydrolysed into ADP, at which point the vulnerable *cis* assembly is dismantled by the binding of ATP on the opposing ring. The products of events in one ring are discharged by the initial stage of the same events in the opposing ring. The molecular events associated with ATP binding, ATP hydrolysis and peptide binding must be described more fully to provide a structural context for a definitive understanding of chaperonin-assisted protein folding. □

Methods

Protein expression and purification. Full-length wild-type GroEL and GroES were cloned separately from the *Escherichia coli* genome using the polymerase chain reaction (PCR). Both coding frames were sequenced to ensure that mutations had not occurred. These DNA segments were subsequently inserted into a pET11a (Novagen) expression vector and transformed into *E. coli* strain

BL21(DE3). Growth, expression and collection were the same for both GroEL and GroES. Except where noted, all experiments were performed at room temperature. Briefly, the protein expression was induced with 1 mM IPTG at an absorbance of 1.0 at 600 nm (A_{600}) and cells were collected 3 h after induction. Cells were lysed by sonication at 4 °C in 50 mM Tris-HCl (pH 7.5), 10 mM $MgCl_2$, 1 mM DTT, 0.5 mM KCl, 1 mM EDTA, 0.05% NaN_3 and 50 $\mu g\ ml^{-1}$ PMSF (buffer A). The lysate was cleared by ultracentrifugation. GroEL was purified by sequential column chromatography on DEAE-Sepharose CL-6B, Superdex-200 and Mono-Q (Pharmacia). GroES was purified by sequential column chromatography on DEAE-Sepharose CL-6B, SP Sepharose Fast Flow and Mono-Q (Pharmacia). The typical yield from this purification scheme was ~200 mg of pure GroEL and ~50 mg of pure GroES per litre of culture. Complexes of GroEL and GroES were prepared by mixing 30 mg of GroEL with 5 mg of GroES in 25 ml of buffer A plus 25 mM ADP at 37 °C for 5 min. The sample was then concentrated to about 2 ml and applied to a Superdex-200 (Pharmacia) column that had been equilibrated with buffer identical except that it contained 50 μM ADP. Pure GroEL₁₄/GroES₇ complex (M_r ~910K) eluted in a peak distinct from excess uncomplexed GroES₇ (M_r ~70K). Results from SDS gel electrophoresis show that the components of the final GroEL–GroES complex are approximately in a GroEL subunit: GroES subunit ratio of 14:7.

Crystallization and data collection. GroEL–GroES–(ADP)₇ complex crystals were grown at 18 °C. Crystals of different size and shape were obtained in a variety of combinations of polyethylene glycol and salt. Large, well-formed rectangular crystals were obtained using the hanging-drop method with PEG3000 and sodium glutamate as precipitants. The reservoir consisted of 12% PEG3000, 0.25 M sodium glutamate, 100 mM cacodylic acid (pH 5.5); drops of 6–12 μl contained a 1:1 mixture of 15 $mg\ ml^{-1}$ protein and reservoir solution. Microseeding techniques were used to reproducibly obtain single crystals ranging in size from $0.04 \times 0.2 \times 0.2\ mm^3$ to $0.1 \times 0.5 \times 0.5\ mm^3$. The crystals were suspended in small nylon loops at the end of mounting pins, dipped in 20% PEG3000, 20% ethylene glycol, 0.25 M sodium glutamate, 100 mM cacodylic acid (pH 5.5) for a few seconds to ensure the entire crystal was coated with the freezing solution, and flash-frozen using a nitrogen stream at 110 K. The frozen crystals were subsequently transferred to liquid propane and stored in liquid nitrogen. Crystals belong to the space group $P2_12_12$ with cell constants $a = 255\ \text{\AA}$, $b = 265\ \text{\AA}$, $c = 184\ \text{\AA}$. The asymmetric unit contains one GroEL₁₄–GroES₇–(ADP)₇ complex with a solvent content of 65%. Data were collected at Brookhaven National Laboratory on beamline X25 ($\lambda = 0.950\ \text{\AA}$) using a MAR imaging plate detector system. The diffraction of the crystals was anisotropic, with diffraction to $3.0\ \text{\AA}$ along the c axis and $2.7\ \text{\AA}$ in the other two directions. The crystals also suffered from radiation damage despite liquid-nitrogen cooling. Data processing and reduction was performed using DENZO and SCALEPACK programs⁵⁰.

Structure determination and refinement. Self-rotation (using X-PLOR⁵¹) searches indicated an unambiguous 7-fold rotation axis nearly parallel to the c axis. The tetradecamer from the refined unliganded GroEL crystal structure⁵² was used as a search model for molecular replacement. The orientation and position of the 14 subunits of the GroEL was found by rotation (12.5σ) and translation (25.6σ) searches at $4.5\text{-}\text{\AA}$ resolution (X-PLOR⁵¹). Packing analysis based on the orientation and position of GroEL revealed that the GroEL–GroES complexes stack one upon another along the c axis. As the 7-fold axis is nearly parallel to the axis c , the dimension of complex along its 7-fold axis would be roughly the length of c . A cylindrical molecular envelope was constructed with the radius of the GroEL molecule and the length of the c axis. The electron density inside the envelope was averaged around a proper 7-fold axis using phases calculated with the molecular replacement model at $4.5\ \text{\AA}$. A clear ‘bullet-shaped’ boundary was evident in the averaged map for the assembly similar to the image observed in previous electron-microscopy studies³⁷. Starting at $4.5\ \text{\AA}$ and proceeding to $3.5\ \text{\AA}$, phases were extended and improved by 20 steps of non-crystallographic symmetry (n.c.s.) averaging/solvent flattening/histogram matching about the same 7-fold axis using the DPHASE program (G. VanDyne, unpublished). The result was a map that showed connected backbone density and the outline of the subunits. The electron-density map was then skeletonized using the program O⁵³ and edited to avoid overlapping between subunits. An envelope that included only one protomer (one GroEL subunit from each ring and one GroES subunit) was

created for subsequent averaging by centring spheres of $5\text{-}\text{\AA}$ radius on the edited skeleton atoms. The new envelope was applied to a $12\text{-}\text{\AA}$ electron-density distribution calculated with random phases, using a set of general superimposition matrices obtained from the molecular replacement solution and adjusted with rigid-body refinement. Phases were refined and gradually extended to $3.0\ \text{\AA}$ by molecular averaging and solvent flattening using RAVE⁵⁴. As the resolution was extended, the envelope was periodically edited to include emerging molecular features and the matrices were updated. The resulting map showed clearly the entire fold of GroES and the domain rearrangements of GroEL. Models of GroES⁴⁰ and the intermediate and apical domains of GroEL⁵¹ were fitted to the density to produce a much more accurate envelope by centring spheres of $5\text{-}\text{\AA}$ radius on all the backbone atoms (GroES coordinates were generously provided by J. Hunt and J. Deisenhofer). The phase refinement and extension procedure was repeated, resulting in an electron-density map from which the entire structure could be traced. The apical domain, suspected for functional reasons to be inherently flexible and poorly ordered, shows defined electron density for all main-chain atoms but poor densities for some side-chain atoms. The C-terminal 23 residues were not visualized. The model was refined with positional refinement and simulated annealing using program CNS (A. T. Brunger, personal communication), which combines torsion angle dynamics⁵⁵ with a maximum-likelihood target⁵⁶. The refinement was interspersed with manual rebuilding using O⁵³. The refinement was monitored using the free R -factor (5% of the data were saved for free R -factor calculation). The refinement was begun with strict n.c.s. constraint. As the refinement proceeded, protomers were allowed to deviate slightly from their RAVE-defined arrangements. Equatorial and intermediate domains were more tightly restrained by n.c.s., and apical domains and GroES molecules were allowed more generous deviations. A flat bulk solvent correction and an overall anisotropic B -factor scaling was applied to the data after the R -factor reached 30%. The refinement statistics are summarized in Table 1. The C-terminal 23 residues were not modelled in the structure.

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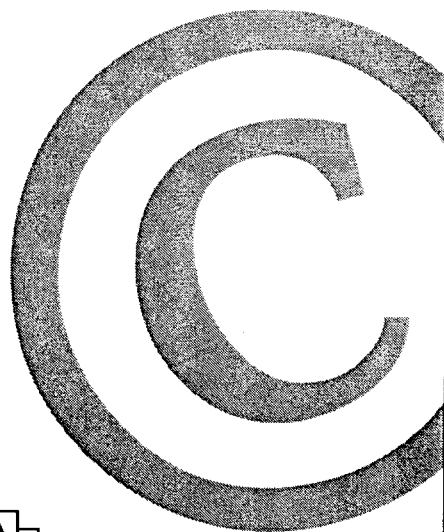
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Faint X-ray sources in the core of the globular cluster M28

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Globular clusters, the most ancient stellar groups in our galaxy, are known to contain bright X-ray sources, faint X-ray sources and millisecond pulsars. The bright X-ray sources are neutron stars accreting matter from a companion star¹, and the millisecond pulsars are believed to be descendants of these sources². But the origin of the faint X-ray sources remains unclear. Here we report satellite-based X-ray observations of the globular cluster M28 which reveal two faint X-ray sources; an extended source slightly offset from the centre of the cluster, and a point source. The point source pulsates with the same period as a well-known^{3,4} 3-millisecond pulsar in M28. The nature of the extended source is more puzzling, however, and its spatial and spectral properties permit a range of plausible models. We argue that this source is either a collection of low-luminosity accreting neutron-star binaries or a synchrotron nebula powered by a recent outburst of energy from an unknown source. Sensitive optical and X-ray observations should be able to distinguish between these two possibilities.

The globular cluster M28 was observed with the High Resolution Imager (HRI) aboard the X-ray observatory satellite Rosat⁵ between 18 March and 21 April 1995. The on-source integration time was 41.7 ks. Our HRI image shows that the X-ray source RX J1824.5 – 2452 discussed earlier^{6,7} breaks up into separate sources (Fig. 1): a point source RX J1824.5 – 2452P and an extended source RX J1824.5 – 2452E. No other source is seen in the 1-degree field of view of the HRI. The count rate within an aperture of 7 arcsec centred on each source is $3.7 \times 10^{-3} \text{ s}^{-1}$ for RX J1824.5 – 2452P and $7.7 \times 10^{-3} \text{ s}^{-1}$ for RX J1824.5 – 2452E. The background count rate, normalized to the same aperture radius, was found to be $0.2 \times 10^{-3} \text{ s}^{-1}$. The radius of the aperture is a compromise choice in view of the small separation, 9.3 arcsec, between the two sources. The sum of the two source count rate is well determined; the individual count rates depend on the details of the photometry. We conservatively estimate an uncertainty of 20% in the individual count rates.

M28 was also observed by the X-ray satellite ASCA⁸. The analysis of these data is reported elsewhere⁴. The angular resolution of ASCA compared to that of the HRI is poor, a few arcminutes, instead of a few arcseconds. Thus the two sources discussed above are seen as only one source in the ASCA data. However, ASCA has broad-band coverage (1–10 keV) and is well suited for spectroscopy. Pulsations at the period of the 3-ms radio pulsar B1821 – 24 (ref. 3) were detected in this data. We carried out a similar search for the HRI data and confirm the X-ray pulsations seen in the ASCA data (Fig. 1). By tagging each HRI photon detection with its corresponding phase bin, we created two images: one when the pulsar is 'on' and the other when it is 'off' (Fig. 1). These two images clearly demonstrate that RX J1824.5 – 2452P is indeed the pulsar.

The ASCA spectrum can likewise be separated into two parts⁴. The ASCA spectrum of the sum of the pulsed and unpulsed components is well described by a power-law spectrum and a neutral hydrogen column density, $N_{\text{H}} = 2.8 \times 10^{21} \text{ cm}^{-2}$. This is

consistent with the value extrapolated from the observed dispersion measure to the pulsar. As can be seen from Figs 1 and 2, the unpulsed signal is entirely due to RX J1824.5 – 2452E. Our ASCA spectrum of the non-pulsed signal may be well described by a power-law model with photon index 1.94 ± 0.12 , or an optically thin thermal model with $kT = 4.2_{-1.0}^{+1.6}$, or a thermal bremsstrahlung model with $kT = 6.0_{-1.0}^{+3.5}$, where T is temperature. We used the program PIMMS (Portable Interactive Multi-mission Simulator), developed at the Goddard Space Flight Center, to convert the HRI photon rates to flux. We applied the ASCA spectral models to the pulsed and unpulsed signals and obtained the following unabsorbed fluxes in the ROSAT HRI energy range of 0.1–2.4 keV:

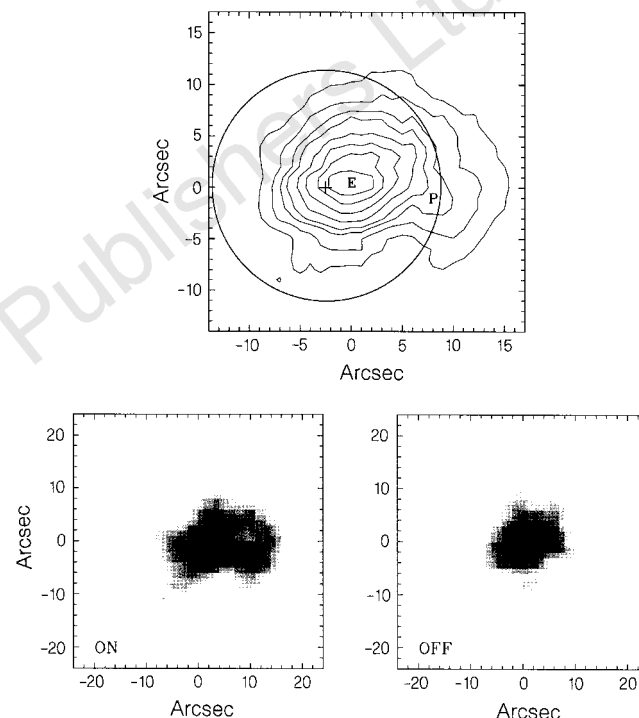


Figure 1 Rosat HRI image of the sources in M28. The HRI photon events were binned into an image with a pixel scale of 1 arcsec per pixel. The image was then convolved with a two-dimensional gaussian function with $\sigma = 2$ pixels. The resulting spatial resolution of the image is quite close to the width of the point-spread function, 2.2 arcsec, derived from ground-based calibration and in-flight observations²⁰. The orientation in all images is north up and east to the left. Top panel, contour plot created from 41.7-ks of integration time. The source in the core of M28 clearly shows a resolved structure. The positions of RX J1824.5 – 2452P (P) and RX J1824.5 – 2452E (E) are marked. The cross and the circle mark the optical centre and core radius of M28; these were obtained from refs 21, 22. For this plot the X-ray image has been shifted so that the pulsed X-ray source RX J1824.5 – 2452P coincides with the radio position of pulsar B1821 – 24. A horizontal bar indicates the image scale. The fact that HRI events are time-tagged (to a precision of 60 μs) allows us to study pulsations from the 3-ms radio pulsar known to be in the core of this cluster. To this end we restricted the analysis to the first 29.3 ks of data; these data were obtained over 70 h. The radio barycentric period at the start of the Rosat observation (JD 2,449,795.0) was extrapolated from the radio ephemerides of Cognard *et al.*²³ as 3,054,314,886,818 ms. Over the 70 h of observing time, this period is expected to change by <0.5 ps. The folded data were subject to a Z^2 test²⁴; we found $Z^2_0 = 34.4$. Such a high value is found by chance at a probability level of 10^{-6} . X-ray pulsations from the pulsar have clearly been detected. See Fig. 2 for the resulting pulse profile. The bottom panels compare the two sources during the on-phases and the off-phases of pulsar B1821 – 24. For the on-phase image, photons from phase 0.3–0.4 and phase 0.8–0.9 were combined. For off-phase image, photons from phase 0.0–0.2 were combined with photons from phase 0.5–0.7. Here it is clear that RX J1824.5 – 2452 consists of two independent sources.

$S_p = 3.9 \times 10^{-13} \text{ erg cm}^{-2} \text{ s}^{-1}$ and $S_E = 1.1 \times 10^{-12} \text{ erg cm}^{-2} \text{ s}^{-1}$ (here and below the subscripts P and E stand for the pulsar and the extended source, respectively).

What is RX J1824.5 – 2452E? It is clearly extended, which renders unlikely the hypothesis that this is a background active galactic nucleus (AGN) or a foreground interloper (a star with an active corona). The extended emission could possibly be due to a galaxy or a cluster of galaxies. The expected total number of extragalactic sources is 3 per square degree for $S > 10^{-13} \text{ erg cm}^{-2} \text{ s}^{-1}$ (ref. 9). According to ref. 10, most of these sources are due to AGN with the galaxies contributing 6% and clusters a mere 1%. Galaxies dominate only at flux levels well below $10^{-13} \text{ erg cm}^{-2} \text{ s}^{-1}$. Thus, from a statistical point of view, it is unlikely that RX J1824.5 – 2452E is emission from a galaxy. The close location of RX J1824.5 – 2452E to the centre is suggestive of the source being a cluster. Accordingly, we now consider models in which RX J1824.5 – 2452P is an X-ray source within the globular cluster M28.

Adopting a distance to RX J1824.5 – 2452P of 5.1 kpc (ref. 11), we derive isotropic luminosities (in the Rosat band) of $L_p = 1.1 \times 10^{33} \text{ erg s}^{-1}$ and $L_E = 3.3 \times 10^{33} \text{ erg s}^{-1}$. A plausible model for RX J1824.5 – 2452E under the assumption that this source is in M28 must account for the following three observations: (1) the source is extended, $\sim 0.26 \text{ pc}$ at the distance to M28; (2) the source is within the core radius but not located at the centre of M28; and (3) the X-ray spectrum of the source is hard. We now consider two classes of models for RX J1824.5 – 2452E: a nebular origin and a stellar origin.

In the first model, the extended emission is from a nebula. Krockenberger and Grindlay¹² have noted diffuse X-ray emission in the globular cluster 47 Tucanae; not much is known about its spectral properties. These authors explain the lack of a corresponding radio nebula by suggesting that the X-ray emission arises from Compton scattering of optical photons by a pool of energetic particles. The model does not explain how the energetic particles are confined and we consider this as a fatal limitation of the hypothesis. A more conventional explanation is that RX

J1824.5 – 2452E is a synchrotron nebula similar to the Crab nebula. The X-ray spectrum of the Crab nebula is well described by a power law with a photon index of 2; This index becomes 1.3 below the so-called 'break frequency', ν_B . For RX J1824.5 – 2452E the spectral flux density at frequency $\nu_B = 3 \times 10^{17} \text{ Hz}$ (corresponding to photons with energy of 1 keV) if $f_X = 0.4 \mu\text{Jy}$. Thus the expected flux at a frequency ν (with $\nu < \nu_B$) is $f_B(\nu/\nu_B)^{-0.3}$; here $f_B = f_X(\nu_B/\nu_X)^{-1}$ is the spectral flux at the break frequency. There is no radio counterpart to this nebula at $\nu = 1.4 \text{ GHz}$, $f < 1 \text{ mJy}$ (ref. 13). Thus ν_B must be greater than $\sim \nu_X/20$. In contrast, ν_B for the Crab and other pulsar powered nebulae is in the infrared or radio frequencies. Using the above value of ν_B we derive the luminosity of the nebula across the entire electromagnetic spectrum. The standard synchrotron nebula model¹⁴ relates this to the energy in particles and nebular magnetic field strength. The total energy is related to the integrated luminosity of the nebula; using the above upper limit at 1.4 GHz, we obtain an upper limit to the integral luminosity of $8 \times 10^{33} \text{ erg s}^{-1}$. The corresponding upper limit to the total energy in particles is 10^{45} erg and the equipartition nebular magnetic field strength is below $87 \mu\text{G}$. Energetic electrons gyrate in this magnetic field and lose energy. The lifetime of the electrons which produce the photons of energy $h\nu_B$ is a rough measure of the age of the nebula; we find this to be greater than 200 yr. However, this age is much smaller than the $3 \times 10^7 \text{ yr}$ age of the 3-ms pulsar¹⁵. This discrepancy is so large that we abandon the model in which the nebula is powered by the 3-ms pulsar. Preserving the synchrotron nebula model requires that, $\sim 200 \text{ yr}$ ago, there was an energetic event in the core of M28 with a total energy of 10^{45} erg .

In the stellar model RX J1824.5 – 2452E is due to a number of faint X-ray sources. The sources could be accreting binaries containing white dwarfs (cataclysmic variables, CVs) or neutron stars (low-mass X-ray binaries (LMXBs) of low luminosity). HRI observations of one of the nearest globular clusters, NGC6397, reveal the presence of faint sources with X-ray luminosity $L_X \approx (2-10) \times 10^{31} \text{ erg cm}^{-2} \text{ s}^{-1}$ (refs 16, 17). Grindlay *et al.*¹⁸ have reported optical data and argue that these faint sources are magnetic CVs. A few tens of such CVs could then account for RX J1824.5 – 2452E. However, the X-ray spectra of nearby magnetic CVs are very hard with a characteristic temperature of 30 keV, whereas the characteristic temperature of RX J1824.5 – 2452E is 5 keV. This then leads us to consider that the faint X-ray sources are faint LMXBs, that is, accreting neutron-star systems. An example of such a system is Centaurus X-4 which has $L_X \approx 2 \times 10^{32} \text{ erg s}^{-1}$. The ASCA spectrum of Cen X-4 can be explained by a combination of 0.3-keV black body and a long hard-spectrum tail¹⁹. It is plausible that the blackbody component arises from the heated surface. Depending on the accretion history, it is possible to have the hard-spectrum tail dominate over the blackbody component. The non-central location of RX J1824.5 – 2452E may seem to be a problem. However, we do not consider this to be the case for two reasons. First, the non-central location could be due to chance fluctuations. Second, the displacement is small and, given the statistics of a small number of photons, it is entirely possible that the displacement from the centre is not statistically significant.

Thus we have developed two plausible models for RX J1824.5 – 2452E: a synchrotron nebula model powered by an energetic event that took place $\sim 200 \text{ yr}$ ago, or collective emission from a dozen faint LMXBs. The two possibilities are distinguishable. The nebula may be detectable at optical frequencies, $\nu_0 \approx 6 \times 10^{14} \text{ Hz}$, with a surface brightness of $26 \text{ mag arcsec}^{-2}$. The LMXB model would be favoured if any variation in the X-ray flux of RX J1824.5 – 2452E were to be detected; this is because most LMXBs are variable sources.

Note added in proof: J. E. Grindlay (personal communication) has suggested that the spectral properties of the population of faint sources discussed here are also consistent with a population of so-called DQ Her CVs. □

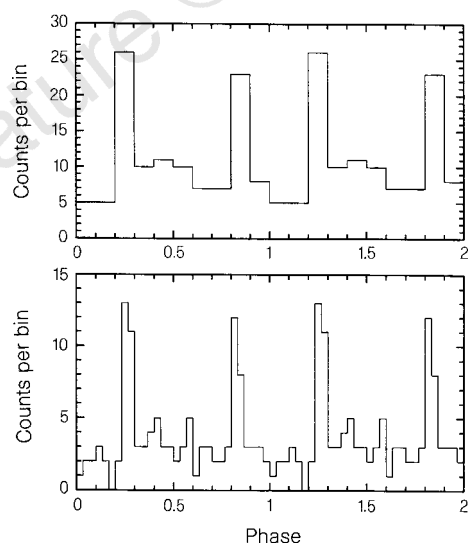


Figure 2 X-ray pulse profile of pulsar B1821-24 taken with the Rosat HRI. See Fig. 1 legend for details of the timing analysis. A total of 112 photons was detected within 7 arcsec of the radio position. In the top panel, the data are shown at a resolution of 10 phase bins; in the bottom panel, the same data are shown at a resolution of 30 phase bins. The profile shows two very narrow peaks. The calibration of the Rosat clock does not allow us to phase-reference, with the required degree of precision, the X-ray light curve with the radio pulse profile. Therefore we choose an arbitrary zero phase point.

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Turbulent drag reduction by passive mechanisms

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In many situations involving flows of high Reynolds number (where inertial forces dominate over viscous forces), such as aircraft flight and the pipeline transportation of fuels, turbulent drag is an important factor limiting performance. This has led to an extensive search for both active and passive methods for drag reduction¹. Here we report the results of a series of wind-tunnel experiments that demonstrate a passive means of effectively controlling turbulence in channel flow. Our approach involves the introduction of specified patterns of protrusions on the confining walls, which interact with the coherent, energy-bearing eddy structures in the wall region, and so influence the rate at which energy is dissipated in the turbulent flow. We show that relatively small changes in the arrangement of these protrusions can alter the response of the system from one of drag decrease to increased mixing (drag enhancement).

Experiments were carried out in a fully developed turbulent channel flow of half-height $h = 2.815$ cm, width 75 cm and length 8.5 m. (A description of the channel may be found elsewhere².) The range of Reynolds numbers (Re) of the channel, based on centre-

line velocity, U_0 , was $1.5 \times 10^4 < \text{Re} = U_0 2h/\nu < 4 \times 10^4$, where ν is the kinematic viscosity. The flow therefore falls into the realm of fully developed turbulence downstream of the entry region³. By convention, such turbulent flows are viewed as having a central core flow which is referred to as the outer region, in contrast with the layer adjacent to the wall, referred to as the inner region. In the former region the characteristic length scale is the channel half-height h which, with U_0 , defines a 'turnover' timescale, $\tau_0 = h/U_0$. Both h and τ_0 represent estimates of scales over which correlations are lost. Further, in the outer region viscosity plays an asymptotically small role and the mean velocity, $U(y)$, scales as $U_0 - U(y) = U_0 f(y/h)$, where y is the wall-normal distance. The inner velocity scale is the friction velocity, u_* , defined by the wall friction, $u_*^2 = \nu [\partial U / \partial y]_{y=0}$. The inner length scale is then given by $l_* = \nu/u_*$, and the mean velocity scales as $U(y)/u_* = g(y/l_*) = g(y_*)$, referred to as the law of the wall. u_* defines a second Reynolds number, $\text{Re}_* = u_* h/\nu$, on which basis $750 < \text{Re}_* < 2,000$ in our experiments.

Both $f(y/h)$ and $g(y_*)$ are regarded as being universal functions. Postulation of an overlap region^{4,5} leads to the 'log-law', $U/u_* = \kappa^{-1} \ln y_* + B$ where κ , the von Karman constant, and B are empirically determined. Evidence of the log-law, in an investigation of pipe flow over three decades of Re (ref. 6), indicates a logarithmic overlap region at high Re (ref. 7).

Several features of the flow show universality. In particular fluctuations in the direction of the flow (u_{rms}) and turbulence production ($-\overline{uv}dU/dy$) peak at $y_* \approx 14$. Here u and v denote velocity fluctuations in the streamwise and wall-normal directions, respectively, and $\overline{uv}$, the Reynolds stress, is the temporal covariance. Of particular importance is the universal presence of 'streaks' in the wall region^{8,9}, which refer to counter-rotating rolls, aligned in the stream direction, that confer on the flow a (statistically) quasi-periodicity in the transverse direction of wavelength $\lambda \approx 100l_*$ (ref. 10). ('Transverse' here indicates the direction across the width of the channel.) Simulations indicate that these are tubular structures, of positive or negative vorticity with respect to the stream, which extend in the stream direction for as much as $1,000l_*$ (ref. 11). Simple scaling arguments suggest that roll spacing should grow linearly with wall-normal distance, and this has been verified experimentally². A variety of mechanistic theories have been suggested to explain the presence of streaks^{12–14}, with no general agreement. More recently a statistical theory¹⁵ suggests that the rolls are the result of an inverse cascade^{16,17}; that is, instead of the normal cascade of turbulence to smaller scales, this cascade takes the flow to larger scales. The spacing is then well estimated by balancing turbulence production and the rate of ejected energy from the wall region.

It has long been known that these structures undergo a rapid cycle of events culminating in their eruption from the wall region, known as 'bursts', which results in slow-moving wall fluid entering the outer region and fast-moving outer fluid entering the wall region; the latter are known as 'sweeps'^{18–20}. These relatively infrequent events account for a significant fraction of wall drag. One view of this phenomenon is that if coherent structures could be stabilized drag would be reduced. (Or from a more general perspective, stabilized coherent structures present a barrier to the cascade to small scales, and hence their maintained presence would impede the rate of energy loss.) Explanations of drag reduction by means of riblets use this viewpoint²¹. Riblets (wall grooves aligned with the stream, with transverse wavelength and height $\sim 15l_*$), which in physical experiments can produce drag reductions of $\sim 6\%$ (refs 21, 22), and $\sim 4\%$ in simulations^{23,24}, are thought to restrain the movement of the rolls and therefore maintain their coherence. The methods of turbulence control presented here differ radically from this approach.

Analysis of several numerical simulations^{25,26} reveals that turbulent energy resides primarily in stream-independent (roll) modes. Further, this analysis also revealed the presence of propagating plane

wave modes travelling downstream at an almost fixed speed. These are possibly related to the sublayer waves observed by Morrison *et al.*²⁷. The waves, which are obliquely orientated with respect to the direction of flow, undergo triad interactions with the roll modes. The most intense of these interactions take place with waves which propagate in a range of angles, 45° – 65° , to the right or left of the stream direction and have length scales comparable to the roll size. A recent numerical investigation (carried out in the simplifying framework of a minimal channel²⁸) gives further evidence of the importance of the interaction between selected propagating modes and the energy-bearing roll modes for the cycle of events which occur in the flow²⁹. (For a recent review see ref. 30.) The importance of these interactions has been further underlined in an earlier investigation in which the relevant propagating waves received regular phase randomization (a workless change) during the course of the simulation³¹. This numerical experiment led to drag reductions well in excess of 50%. This drag reduction is comparable to that found in low-concentration polymer additive experiments^{32,33}; there was a close resemblance in all statistical measures for the case of polymers and the simulation.

This series of observations suggested that management of wall turbulence might be achieved with wall protrusions, chosen to excite appropriate propagating modes. This was tested in the channel shown in Fig. 1a; details are given in the figure legend. Of the variety of wall patterns that were tested, two are shown in Fig. 1b and c. Here we will focus on experiments performed on these two patterns, and comment briefly on other tested patterns.

The entrant flow passed through $157h$ of the channel (this

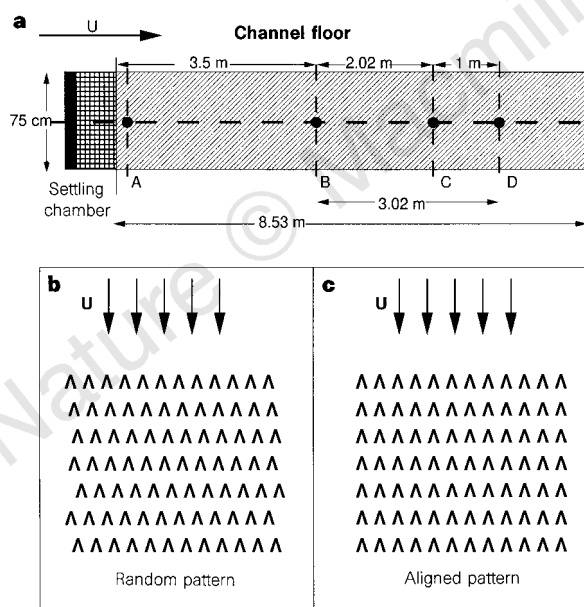


Figure 1 a, Plan view of the floor of the channel used in these experiments. The region shown black is 0.3 m long and represents the settling chamber into which flow enters vertically through 4-mm holes, both in the floor and the ceiling of the channel. The following 0.9 m (squares) is a rough surface. The remainder of the channel (hatched) is either covered with the patterns shown in **b** or **c**, or left smooth. The floor to ceiling height is 5.63 cm, whether the floor is covered with patterns or not. As the protrusions of the pattern, on average, effectively shorten the height, our measurements are conservative. (Surface area is increased by ~5%.) The black circles on the centre line show pressure-tap locations. The mid-station, B, houses a hot-wire system which yields wall-normal and transverse measurements. **b**, **c**, Protrusion patterns on channel floor. Pattern **b** is obtained from the regular array shown in **c** by random shifts of rows. Each vee has width in the transverse direction of $200/\lambda_0$, period in the transverse direction of $260/\lambda_0$ and period in the flow direction of $300/\lambda_0$. The height of the vees, perpendicular to the floor, is $5/\lambda_0$ – $6/\lambda_0$.

includes the rough surface) before reaching the mid-station, B. Hot-wire measurements were taken at station B, and pressure measurements were made at the other stations shown in Fig. 1a. Channel geometry and Reynolds-number range are comparable to other reported experiments^{34–36}. However, although our channel was significantly longer than in these other studies, it fell short of the length of the channel used by Hussain and Reynolds³⁷. A small along-stream variation existed beyond B (see below), as implied by Hussain and Reynolds³⁷.

A series of baseline measurements, for later comparison, were carried out in the absence of any applied patterns, and all mention of drag increase or decrease are relative to this baseline data set. Figure 2 shows the Reynolds-number ($= U_0 2h/\nu$) dependence of the unaveraged raw data for the friction coefficient $C_f (= 2u_\tau^2/U_0^2)$. Individual data sets showed little noise and each is well fitted by a power law. The average over the four data sets conforms to the power law, $C_f \approx 0.0254/\text{Re}^{0.165}$, which compares with $C_f \approx 0.0269/\text{Re}^{0.178}$ found by Hussain and Reynolds, who averaged over many experiments performed over a significantly larger Reynolds-number range. (The four data sets shown in Fig. 2 are actually quite close; the lower two sets of points are fitted by $C_f \approx 0.0253/\text{Re}^{0.167}$.) The vertical variation, $< \pm 3\%$, seen in the plot was due to uncontrollable ambient conditions. Transducer calibrations indicated a sensitivity of 0.5%, and pressure variations due to temperature changes caused a $\pm 0.8\%$ error, resulting in a net $\pm 1.3\%$ error in the friction measurements. Mean velocity and Reynolds stress measurements performed at B confirmed the friction values shown in Fig. 2. These and other turbulence statistics will be reported elsewhere.

Figure 2 also shows the results for C_f when the randomly shifted row pattern (Fig. 1b) is applied to the floor of the channel. C_f for the three data sets for the patterned floor all lie below their smooth-floor counterparts. The average of these, $C_f \approx 0.0248/\text{Re}^{0.172}$, represents a drag reduction of ~10% against the smooth-floor average over the range shown. The peak drag reduction, data points represented by 'x's over the smooth-floor average, exceeds 12.5%. The C_f results shown in Fig. 2 and quoted here are based on pressure drops measured between B and D, a 3.02-m span. C_f , when measured between B and C, a 2.02-m span, differed by 2% from these values. Although this lies within experimental error we took it as an indication of residual stream dependence.

The pattern was chosen (on the basis of the above-cited references) to produce waves which interact maximally with the energy-bearing roll modes. The goal was to prevent formation of the rolls, and to break them up if formed. As a heuristic argument to support this strategy, we can suppose that roll structures have characteristic velocity u_0 , and dimension λ_0 . It might then be supposed that $\tau_0 = \lambda_0/u_0$ is characteristic of the duration of a burst. We denote by fu_0^2 the fraction of the energy contained in roll modes. Flow visualizations imply that as a consequence of a burst the roll goes directly into small scales; on these grounds fu_0^2/τ^0 is a measure of the dissipation rate due to the rolls. On the other hand, if instead the rolls were to follow the cascade route to dissipation, as forced by the protrusions, the estimate changes. We will now assume that the Kolmogorov spectrum is valid for scales below λ_0 , that is, for $\lambda \leq \lambda_0$, $u^2/u_0^2 \propto (\lambda/\lambda_0)^{5/3}$ and therefore $\tau/\tau^0 \propto (\lambda/\lambda_0)^{1/6}$. To model the cascade we will regard an eddy as halving in size for each turnover time. Thus the time $\bar{\tau}$ to fully dissipate the eddy is $\bar{\tau}/\tau^0 = \sum_{n=0}^{\infty} (2^n)^{1/6} (1 - 2^{1/6})^{-1} \approx 8.3$. Thus within this crude depiction of the phenomena, energy degradation is delayed and therefore the dissipation rate due to rolls alone is substantially reduced. From this it can be inferred that the roll modes now carry less energy than in the standard smooth case. The principal premise of this simple modelling of events is that by preventing the creation of the roll 'coherent structures' we delay the time taken until the final loss to dissipation.

Additional measurements support this picture. Burst (and

sweep) frequency ω were measured at the upper (smooth) wall of the channel (the 'ceiling') and the lower (patterned) floor at a wall-normal distance of $30l_*$ and centre-line velocity of 6 m s^{-1} . Following Alfredsson and Johansson³⁸, a burst at the channel ceiling is said to occur when $uv > 4u_{\text{rms}}v_{\text{rms}}$ and $v < 0$ (and a sweep when $v > 0$). Similarly for the channel floor, but for proper comparison the r.m.s. values at the ceiling are used. By these criteria we found the normalized burst frequency, $\omega h/U_0$, at the ceiling to be 0.127 which is comparable to value found in ref. 38. By comparison, the patterned floor had a significantly lower burst frequency of 0.050. (A comparable reduction in the sweep frequency also occurred.) Transverse correlation measurements indicated a streak spacing at the patterned wall 10% greater than at the smooth wall. Also, v_{rms} in the vicinity of the patterned wall was found to be less than at the smooth wall. Increased streak spacing and a diminished v_{rms} are consistent with drag reduction using trace amounts of polymer additives³².

It has recently been shown that if contributions to the average Reynolds stress is separated into roll and propagating mode contributions²⁹, then roll modes are the major contributors near the wall, whereas propagating modes mainly contribute in the core. By interfering with the formation of the roll modes, as the protrusions do, we can diminish the contribution from roll modes.

We performed experiments designed to increase the fraction of energy which reside in roll modes. This was accomplished by using the pattern of protrusions shown in Fig. 1c. In this case, the same 'vee' protrusions (having the same height of roughly $5l_* - 6l_*$) are now aligned as columns in the stream direction. As evidenced from direct hot-wire spanwise transverse measurements, a statistically steady array of counter-rotating rolls was established. Thus, the energy in roll modes was increased. In this case a drag increase well in excess of 20% was obtained. The results are indicated in the upper part of Fig. 2. Unfortunately these data were acquired from pressure-tap locations still showing along-stream variation. As a consequence the results underestimate the effect.

The bursting of the rolls was facilitated by increasing their production. The simple change of misaligning or aligning of the vees, all of which point in the upstream direction, results in a drag reduction of >10% altering to a drag increase in excess of 20%. We have also tested the pattern shown Fig. 1c, but with alternate rows shifted a half-wavelength. This also produced a drag reduction but was less effective than the randomly slid rows of Fig. 1b. Variations

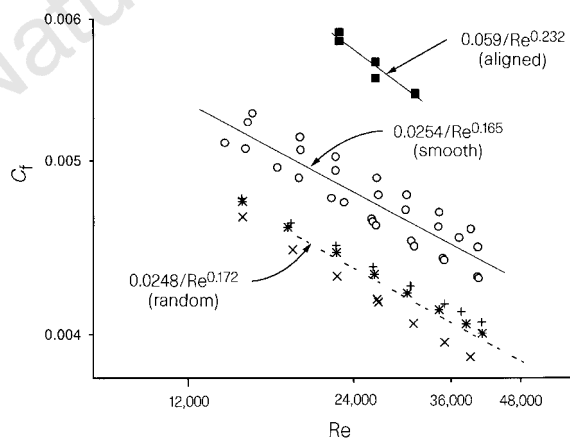


Figure 2 Plot of friction coefficient $C_f (= 2u_\tau^2/U_0^2)$ against Reynolds number $Re (= U_0 2h/\nu)$. Circles, results of four independent experimental runs in a channel with a smooth floor. The continuous straight line is the regression fit to these data. The three sets of symbols in the lower portion of the plot represent three independent experimental runs with the random pattern of Fig. 1b on the floor of the channel. The dashed line is the result of a regression analysis on these data. The filled squares in the upper portion of the figure represent runs carried out on the aligned pattern shown in Fig. 1c. The analytic form of the regression fits are indicated by arrows.

in protrusion heights and the effect of the inclusion of additional permissible vee angles remain to be studied.

A straightforward argument indicates that if both floor and ceiling of the channel are covered with the patterned material, the effect should at least double. Unfortunately, the design of the channel did not allow this and we were not able to pursue this idea. We are redesigning the channel to permit such experiments. Finally, we mention that over the past 15 months, during which we improved our experimental procedures and verified the phenomena, almost without exception each experiment with the randomly patterned floor showed a drag reduction. □

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Large-scale production of single-walled carbon nanotubes by the electric-arc technique

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Single-walled carbon nanotubes (SWNTs) offer the prospect of both new fundamental science and useful (nano)technological applications¹. High yields (70–90%) of SWNTs close-packed in bundles can be produced by laser ablation of carbon targets². The electric-arc technique used to generate fullerenes and multi-walled nanotubes is cheaper and easier to implement, but previously has led to only low yields of SWNTs^{3,4}. Here we show that this technique can generate large quantities of SWNTs with similar characteristics to those obtained by laser ablation. This suggests that the (still unknown) growth mechanism for SWNTs must be independent of the details of the technique used to make them. The ready availability of large amounts of SWNTs, meanwhile, should make them much more accessible for further study.

In our electric arc-discharge apparatus⁵, the arc is generated between two electrodes in a reactor under a helium atmosphere (660 mbar). The cathode was a graphite rod (16 mm diameter, 40 mm long) and the anode was also a graphite rod (6 mm diameter, 100 mm long) in which a hole (3.5 mm diameter, 40 mm deep) had been drilled and filled with a mixture of a metallic catalyst and graphite powder. The arc discharge was created by a current of 100 A; a voltage drop of 30 V between the electrodes was maintained by continuously translating the anode to keep a constant distance (~3 mm) between it and the cathode. Typical synthesis times were ~2 min. As the catalyst we used mixtures such as Ni–Co, Co–Y or Ni–Y in various atomic percentages; these are known to yield a series of interesting carbon nanostructures⁶. The mixture used by Guo *et al.*⁷ during their laser ablation process (Co and Ni, both at 0.6 at.%) did not produce a good yield of nanotubes in our case. However, we found that a mixture of 1 at.% Y and 4.2 at.% Ni gave the best results. In this case we observed (in a total carbon mass of 2 g): (1) large quantities of rubbery soot condensed on the chamber walls; (2) web-like structures between the cathode and the reactor walls (no webs when either Y or Ni were absent); (3) a cylindrical deposit at the cathode's end; and (4) a small 'collar' (~20% of the total mass) around the cathode deposit, as a black, very light and porous but free-standing material.

Within all these products it was possible to observe by scanning electron microscopy (SEM; using a JEOL JSM 6300F instrument) filament-like structures that are more or less dense, depending on where in the reactor they were deposited. The 'collar' deposit was densest; the soot was the least dense. A characteristic SEM image of the collar deposit (Fig. 1) shows large amounts of entangled carbon filaments, homogeneously distributed over large areas (here at least a few square millimetres) and with diameters ranging from 10 to 20 nm. The average length between two entanglement points is several micrometres; we could not identify any filament ends. From several SEM images we estimate the yield of these filaments (with respect to the total volume of the solid material) to be of the order of

80%. Higher magnification confirms the existence of entangled filaments although it is not possible at this stage to identify the nature of the crosslinks.

High-resolution transmission electron microscopy (HRTEM; using a JEOL 4000FX instrument) images of the collar deposit (Fig. 2) show that each filament consists of smaller aligned SWNTs self-organized into bundle-like crystallites with diameters ranging

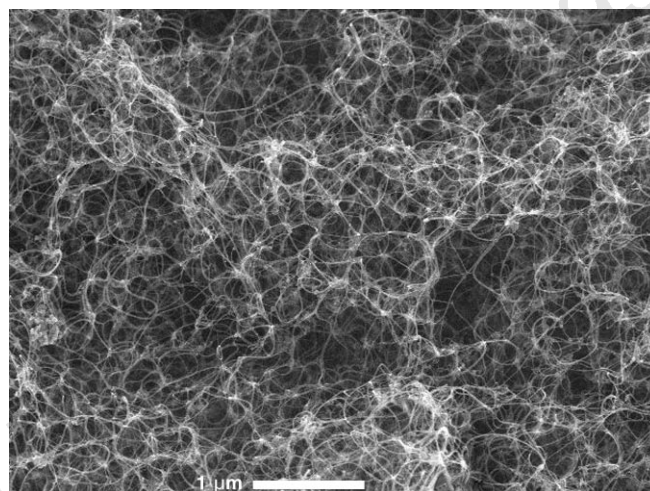


Figure 1 Scanning electron microscopy image of the light and porous material that formed as a collar around the cathode deposit in our apparatus, showing a high density of entangled carbon filaments. Scale bar, 1 μm.

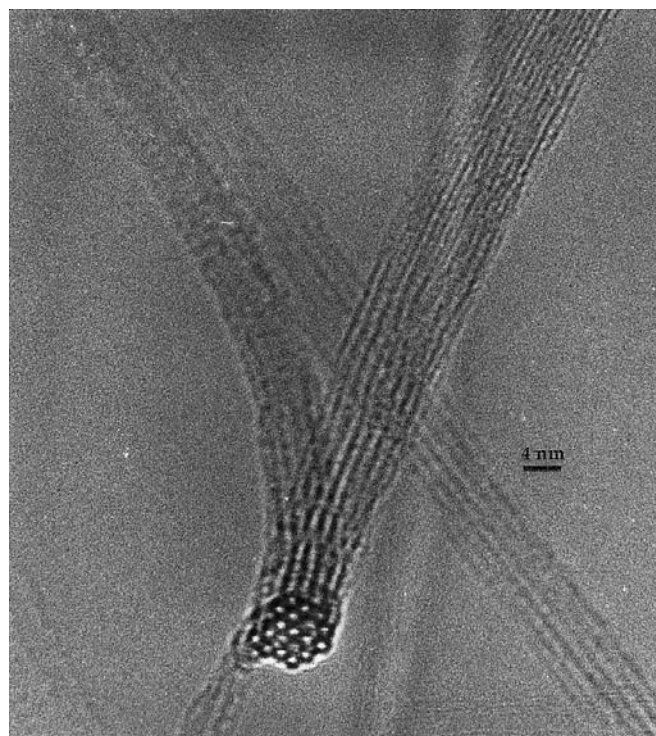


Figure 2 High resolution transmission electron microscopy view of one bundle from the collarette bent in such a way that it is seen edge-on. The bundle has a uniform width and consists of ~20 SWNTs of almost uniform diameter, packed according to a triangular lattice. The focusing conditions are such that the lattice is imaged as a triangular arrangement of strong white dots (which are located at the centres of the SWNTs) on a dark background. Scale bar, 4 nm.

from 5 to 20 nm. As these bundles are often bent, some portions are orientated parallel to the electron beam and are therefore seen edge-on (Fig. 2). The tube diameters are around 1.4 nm, and the average distance between them is 1.7 nm. Evidence for periodic packing is also given by electron diffraction patterns obtained from an assembly of bundles (not shown).

The number of tubes within a bundle is typically of the order of 20, and their section can be either roughly circular (Fig. 2) or elongated along a dense direction of the lattice. Larger-diameter bundles consist mostly of an assembly of smaller bundles separated by twin-like boundaries. The intra-bundle organization is also evidenced by the existence of diffraction fringes whose spacing is directly related to the lattice parameter and depends on the orientation of the crystalline bundle with respect to the electron beam.

Besides the bundles, we see randomly distributed spherical nanoparticles ranging from 3 to 20 nm in diameter, usually embedded in amorphous carbon. These nanoparticles probably consist of the remaining metallic catalyst. The SWNTs pass through

them with no obvious evidence of a direct link. We have seen no other metallic or carbonaceous structures in the deposits.

It is difficult to determine the SWNT diameters accurately from electron microscopy, but the tube configuration can be studied in detail using Raman spectroscopy (Fig. 3). The spectrum exhibits three main zones at low ($140\text{--}200\text{ cm}^{-1}$), intermediate ($300\text{--}1,300\text{ cm}^{-1}$) and high ($1,500\text{--}1,600\text{ cm}^{-1}$) frequencies, and is characteristic of SWNTs (other carbon materials produce other types of spectra^{8,9}). The high-frequency bands can be decomposed into two main peaks around $1,589$ and $1,562\text{ cm}^{-1}$ with shoulders at $1,550$ and $1,530\text{ cm}^{-1}$. These features have previously been assigned to a splitting of the E_{2g} mode of graphite¹⁰. The very-high-resolution spectrum obtained in the low-frequency domain (Fig. 3 inset) shows at least five components at 149 , 164 , 172 , 178 and 183 cm^{-1} . These two spectra were recorded for exactly the same experimental conditions and sample, except that the laser beam explored two different areas. Thus the spectrum in this frequency domain is very sensitive to the sample area investigated, in contrast with that in the range $1,500\text{--}1,650\text{ cm}^{-1}$. In addition, different excitation wavelengths produce different relative intensities of these bands, indicating strong resonance behaviour. According to earlier calculations¹¹, these modes are expected to be of A_g symmetry, and their frequency increases with decreasing tube diameter. Therefore our results clearly reflect a distribution in diameters (or of helical pitch for a given diameter). It can be shown theoretically¹⁰ that only 'armchair' tubes (with diameter and pitch parameters $n = m$ ranging from 6 to 12) generate Raman modes between 750 and 800 cm^{-1} . In Fig. 3 the peaks in the frequency range $300\text{--}1,200\text{ cm}^{-1}$ can be mostly identified as overtones and combinations of lower-frequency modes; but the modes of 750 and 782 cm^{-1} are characteristic of such armchair nanotubes.

X-ray diffraction was performed using an INEL CPS 120 detector in Debye-Scherrer geometry. A small flake from ground collar material was attached to an etched Si(111) wafer moistened with ethyl alcohol. After background subtraction, the data show an intense peak near diffraction vector $Q = 0.43\text{ \AA}^{-1}$ and four weaker peaks up to $1.8\text{ \AA}^{-1}$ (Fig. 4 trace a). These low- Q scattering peaks indicate the existence of a two-dimensional lattice of SWNTs organized in bundles². The width of the peak at $Q = 0.43\text{ \AA}^{-1}$ yields a coherence length of the order of $100\text{ \AA}$, in very good agreement with the average transverse size of the bundles. The region above $Q = 3\text{ \AA}^{-1}$ (Fig. 4 inset) is dominated by two peaks at 3.1 and $3.6\text{ \AA}^{-1}$ which correspond to the (111) and (200) reflections of pure face-centred cubic nickel. The analysis of their shapes (using the Warren-Averbach¹² method) results in a particle size distribution ranging from 2 to 21 nm , with an average size of 11.2 nm . Our X-ray diffraction data are entirely consistent with the observations made by HRTEM.

A direct comparison of the X-ray diffraction data obtained by Thess *et al.*² (Fig. 4 trace b) and by us reinforces the great similarity between both sample characteristics: yields in the range $70\text{--}90\%$, diameters around 1.4 nm , crystalline bundles of a few tens of tubes and only a few isolated SWNTs. Our results therefore imply that there is an unique growth mechanism for these nanotubes, which does not strongly depend on the details of the experimental conditions, but which depends much more on the kinetics of carbon condensation in a non-equilibrium situation. Temperature, and temperature gradients in space and time, must play an important role, as can be seen by the fact that most of the SWNTs were found in a very specific zone of the reactor (a few centimetres around the cathode). Additionally, the use of a second element beside the catalyst Ni or Co during the evaporation process is also critical. In our experiments it is yttrium that strongly favours the growth of SWNTs.

Our electric-arc technique is able to produce gram quantities of well-defined single-walled nanotubes in the form of highly crystalline bundles. It is a cheap method which could be scaled up easily,

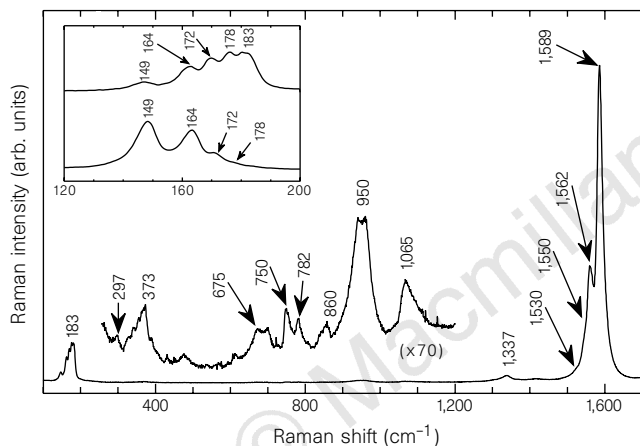


Figure 3 Raman spectrum of SWNTs recorded at room temperature, using an excitation wavelength of 514.5 nm . The intensity in the frequency range between 250 and $1,200\text{ cm}^{-1}$ has been magnified 70 times. Inset, the low-frequency range ($120\text{--}200\text{ cm}^{-1}$) recorded with high resolution at two different spots on the sample.

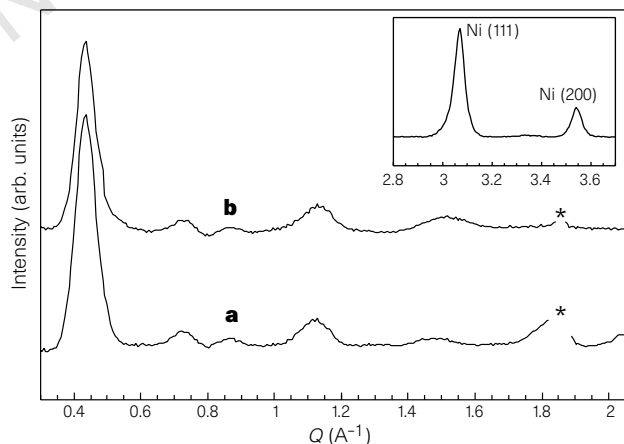


Figure 4 X-ray diffraction patterns at low angle of our collar sample (trace a) and of the sample obtained by the laser ablation technique by Thess *et al.*² (trace b). The graphite peak, due to remaining graphitic particles, has been removed for clarity; its position is shown by an asterisk. Inset, diffraction pattern associated with the Ni nanoparticles.

and so seems to be a very promising alternative to the double-laser-ablation technique². □

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Enantioseparation using apoenzymes immobilized in a porous polymeric membrane

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Chemical separations represent a large portion of the cost of bringing any new pharmaceutical product to the market. Membrane-based separation technologies^{1,2}, in which the target molecule is selectively extracted and transported across a membrane, are potentially more economical and easier to implement than competing separations methods; but membranes with higher transport selectivities are required. Here we describe an approach for preparing highly selective membranes which involves immobilizing apoenzymes within a microporous composite. The apoenzyme selectively recognizes its substrate molecule and transports it across the composite membrane, without effecting the unwanted chemical conversion of the substrate molecule to product. We demonstrate this approach using three different apoenzymes. Most importantly, it can be used to make enantioselective membranes for chiral separations, one of the most challenging and important problems in bioseparations technology. We are able to achieve a fivefold difference between the transport rates of D- and L-amino acids.

Membranes for chemical separations must recognize and transport a specific desired molecule and reject (or transport at lower rates) other molecules present with the desired molecule^{1,2}. Living organisms have solved this problem by incorporating molecular-recognition proteins into lipid bilayer membranes³. By analogy, prototype membranes for chemical separations have been prepared by incorporating molecular-recognition proteins within synthetic membranes^{4,5}. Enzymes might seem to be a likely choice because an enzyme selectively recognizes and binds its substrate molecule. However, enzymes also catalyse a chemical reaction on this molecule, and this unwanted chemical transformation must be subsequently undone in a following chemical step^{6–8}. In addition,

previous enzyme-based membranes for chemical separations have been of the unwieldy (and impractical) liquid-membrane design⁸. For these reasons, although enzymes are very useful in bioreactors^{9,10}, they have not proved useful as molecular-recognition agents in membranes for chemical separations.

It occurred to us that the unwanted chemical conversion of the substrate molecule⁸ in enzyme-based separation could be circumvented by using an apoenzyme as the molecular-recognition agent. Enzymes often require a cofactor to accomplish chemical conversion of the substrate molecule. We will show here that if the apoenzyme (no cofactor) is incorporated into a membrane, the membrane will selectively transport the substrate molecule without the unwanted chemical conversion of this molecule. We use the apoenzymes within a compact, solid-state composite membrane that requires no chemical derivatization of the enzyme.

The membrane design (Fig. 1) consists of a microporous polycarbonate filter that is sandwiched between two thin films of the polymer polypyrrole. The apoenzyme is physically trapped within the pores of the membrane by the polypyrrole films. Apoenzyme is also entrapped within the polypyrrole films. When this (initially dry) sandwich membrane is exposed to a buffer solution, water and electrolyte diffuse through the polypyrrole films and flood the pores. Polypyrrole was chosen as the surface layer material because it is highly permeable to small molecules (for example, the substrate for the enzyme) but does not pass proteins¹¹. In addition, strongly adherent ultrathin polypyrrole films can be grown across the surfaces of the polycarbonate membrane using an electropolymerization method¹¹.

The host membrane used for most studies was a 10-μm-thick filter (Poretics, Livermore, CA, USA) with cylindrical, 400-nm-diameter pores (pore density $1 \times 10^8 \text{ cm}^{-2}$)^{11–14}. The as-received membrane was cleaned by immersion in CH₃CN, and a gold film was then sputtered¹¹ across one of its surfaces. This Au film is too thin (30 nm) to block the pores (Fig. 1), and is used to electropolymerize^{11,15} a polypyrrole film (thickness ~100 nm) across the membrane surface. Plugs of polypyrrole are also deposited a small distance (~1 μm) into the pores (Fig. 1). An Au film was then sputtered across the other surface of the membrane. The pores in the membrane were then loaded with the desired apoenzyme by vacuum filtration of an apoenzyme solution through the membrane¹¹. The apoenzyme solution is applied to the uncoated membrane face so that the solution can freely enter the pores. The uncoated Au film was then used to electropolymerize a polypyrrole film across this surface of the membrane, thus trapping the enzyme within the pores. The completed membrane was mounted between the two halves of a U-tube permeation cell¹². The rate and selectivity of transport of various molecules from the feed half-cell through the membrane and into the permeant half-cell were investigated.

Membranes loaded with alcohol dehydrogenase apoenzyme (apo-ADH) serve to illustrate this new membrane concept. Figure 2a

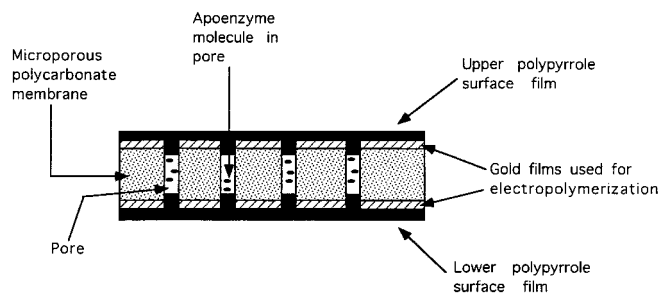


Figure 1 Schematic cross-section of the polypyrrole/polycarbonate/polypyrrole sandwich membrane with the apoenzyme entrapped in the pores. The membrane is drawn as coming out of the plane of the paper. The various components are not drawn to scale.

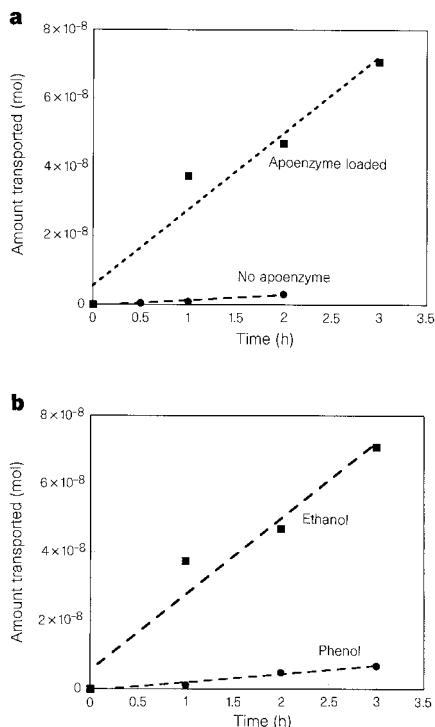


Figure 2 a, Plots of amount of ethanol transported from the feed solution through the membrane and into the permeant solution versus time for a membrane loaded with apo-ADH and for an apo-ADH-free membrane. The feed solution was 0.5 mM in both ethanol and phenol. The slopes of these lines provide the ethanol flux across the membrane. **b**, Plots of amount of ethanol (substrate) and phenol (non-substrate) transported versus time for the apo-ADH-loaded membrane. Feed solution as **a**. The ratio of the slopes provides the selectivity coefficient for ethanol versus phenol transport.

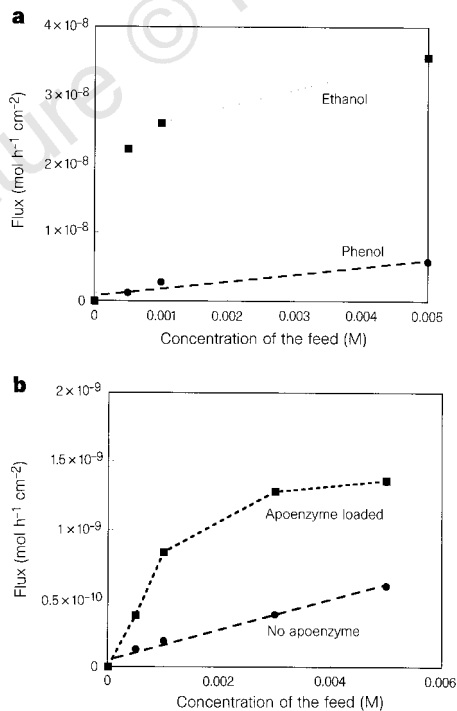


Figure 3 a, Plot of ethanol and phenol flux versus concentration of ethanol and phenol in the feed solution for an apo-ADH-loaded membrane. **b**, Plots of flux of the aldehyde vanillin versus concentration of vanillin in the feed solution for an apo-AddH-loaded and an apo-AddH-free membrane.

compares the ethanol flux for an apo-ADH-loaded membrane with the ethanol flux for a membrane that contained no apo-ADH. The higher flux for the loaded membrane indicates that the apoenzyme is 'facilitating'^{16,17} (see below) ethanol transport. If this is the case, then the flux of a molecule that is not a substrate for the enzyme (phenol) should be lower than the ethanol flux. Figure 2b shows that this is so, and that the selectivity coefficient for ethanol versus phenol transport (the ratio of the ethanol and phenol fluxes) is 9.2. Analogous data were obtained by measuring the ethanol flux from a feed solution containing only ethanol and then measuring the phenol flux from a feed solution containing only phenol. The transport selectivity coefficient obtained in this way was essentially identical (8.7) to the value obtained using the mixed-solution method (9.2) (Fig. 2b). This experiment shows that the transport selectivity coefficient can be obtained by measuring the fluxes from separate solutions or from a solution containing both molecules.

Membranes that contain a chemical species that recognizes and transports a specific molecule are called facilitated-transport membranes^{16,17}. To prove that apo-ADH is facilitating ethanol transport, the concentration dependence of the flux was investigated. If facilitated transport of ethanol is occurring, theory predicts that the ethanol flux should initially increase linearly with feed concentration, and that the flux versus feed-concentration curve should then flatten at higher concentrations^{16,17}. In contrast, the flux versus feed-concentration curve for the non-facilitated molecule (phenol) should be linear because this molecule is transported by simple diffusion^{16,17}. Figure 3a shows that both of these predictions are observed experimentally.

A second apoenzyme (apo aldehyde dehydrogenase, apo-AddH) was used to further confirm the transport mechanism. The concentration dependence of aldehyde (vanillin) flux across apo-AddH-loaded and apo-AddH-free membranes was investigated. The results (Fig. 3b) clearly show that aldehyde transport is facilitated in the loaded membrane. We note that the non-facilitated vanillin flux was always lower than the non-facilitated phenol flux.

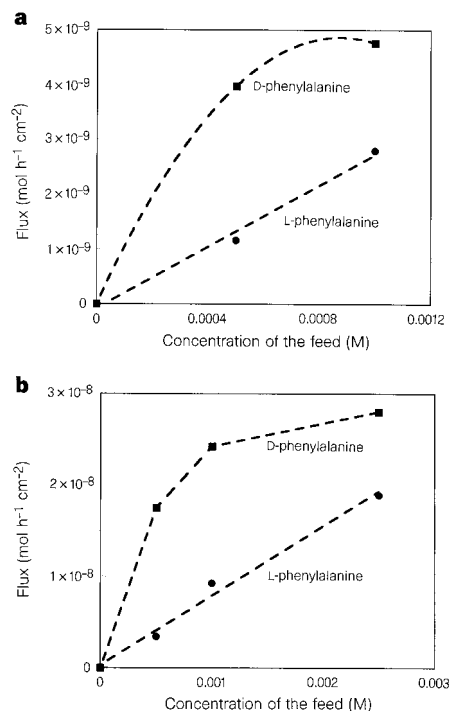


Figure 4 a, Plots of D-phenylalanine and L-phenylalanine flux versus concentration of these enantiomers in the feed solution for an apo-D-AAO-loaded membrane. The membrane had pores of 400 nm diameter. **b**, As **a** but using a membrane with pores of 30 nm diameter (pore density 6×10^8 pores cm⁻²).

These data indicate that the overall flux is influenced by transport in the polypyrrole, with the larger molecule (vanillin) being preferentially impeded. Indeed, all of the fluxes obtained were lower than would be expected if transport were limited by diffusion in the water-filled pores. These results indicate that facilitation is occurring across the polypyrrole layers by apoenzyme entrapped within the polypyrrole¹⁸.

With this proof of concept (and transport mechanism), we turn our attention to the more challenging case of enantioseparations. Enantioseparations are becoming increasingly important because the US Food and Drugs Administration has recently declared that if a drug is chiral, the biological effects of both enantiomers must be determined^{19–21}. D-amino acid oxidase apoenzyme (apo-D-AAO) was used, in conjunction with D-phenylalanine and L-phenylalanine, to explore enantioselectivity in this new type of membrane. Because this enzyme only binds D-amino acids²², we anticipated that membranes loaded with apo-D-AAO would selectively transport D-phenylalanine relative to the L-enantiomer. In addition, we anticipated that the facilitated D-enantiomer would show the characteristic langmuirian flux^{16,17} versus feed-concentration dependence whereas the L-enantiomer would show linear dependence. Both of these results were observed experimentally (Fig. 4).

The data in Fig. 4a were obtained from an aop-D-AAO-loaded membrane that had 400-nm-diameter pores. The maximum D- versus L-phenylalanine selectivity coefficient obtained was 3.3. One route to improve selectivity in a facilitated transport membrane is to shut down the non-facilitated (diffusional) transport of the unwanted species. It occurred to us that this might be accomplished by using a host membrane with smaller-diameter pores.

To test this possibility, we studied the rate and selectivity of transport of apo-D-AAO-loaded membranes with 30-nm-diameter pores (Fig. 4b). The fluxes of both the D- and the L-enantiomers are smaller, but the D- versus L-selectivity coefficient is now larger (maximum value of 4.9). These data not only add further confirmation of the transport mechanism but point to a promising approach for further improvement in enantioselectivity. This value of 4.9 is one of the highest membrane-transport enantioselectivity coefficients to be reported to date^{23–27}, indicating that this is a promising approach for making enantio-selective membranes.

As well as improving selectivity, it is equally as important to improve overall flux. Routes for enhancing flux include using host membranes with higher porosities¹³, preparing thinner surface layers, and using hollow fibres as the host; we note that we have already shown that microporous hollow fibres can be coated with thin skins of poly(*N*-methyl pyrrole)²⁸. In addition, it is important to point out that combinatorial methods have recently been used to synthesize biomacromolecules that selectively recognize and bind a specific target molecule²⁹. Such synthetic molecular-recognition agents could greatly expand the scope of the membrane concept developed here. It is clear that the target molecules that would be most appropriate for separations using such membranes would be high-value organic molecules such as pharmaceuticals. □

Methods

Polypyrrole film syntheses. The first film was grown galvanostatically^{11,15} from a 1:1 water/methanol solution that was 0.2 M in pyrrole and 1 M in NaCl. The membrane area was 8 cm², and an anodic current density of 0.125 mA cm⁻² was applied for 2,500 s. The second film was grown from a CH₃CN solution (0.4 M pyrrole, 0.2 M Bu₄NClO₄) so as not to dissolve the apoenzyme in the pores. We have shown that the enzyme remains active after exposure to CH₃CN. An anodic current density of 1.76 mA cm⁻² was applied for 750 s.

Analytical methods. Ethanol concentration was determined by an enzymatic method using alcohol dehydrogenase and nicotinamide adenine dinucleotide (NAD⁺)^{30,31}. Phenol concentration was determined from its ultraviolet (UV) absorbance at 271 nm. The membrane (2.5 cm dia) was loaded with 3 mg of apo-ADH. That the apo-ADH (and other enzymes) remained active after

loading was proven using standard enzymatic assays^{30,31}. Vanillin concentration was determined from its UV absorbance at 278 nm. The membrane was loaded with 1 mg of apo-ADH. The concentrations of D- and L-phenylalanine were determined from their UV absorbance at 256 nm. Fluxes and selectivity coefficients were evaluated from separate solutions of the enantiomers. The membrane was loaded with 1 mg of apo-D-AAO.

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Torsional oscillations and the magnetic field within the Earth's core

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An estimate of the magnitude and geometry of the magnetic field within the Earth's core would be valuable for understanding the dynamics of the liquid outer core and for constraining numerical models of the geodynamo. The magnetic field down to the core-mantle boundary can be estimated from surface observations by assuming that the mantle is an insulator¹, but such estimates

cannot be further extrapolated into the conducting core itself. The magnetic field within the core has therefore remained largely unconstrained. Here we construct a simple picture of part of the magnetic field within the core by first showing that the fluid flow at the surface of the core is consistent with the presence of two large waves—‘torsional oscillations’ of the type that have been proposed to explain the temporal variation of the magnetic field at the core–mantle boundary. We then use the structure of these waves to calculate a one-dimensional map of the part of the magnetic field that points away from the rotation axis. These results may help distinguish between the different dynamic states proposed for outer-core flow^{2–5} and provide a test for recent numerical models of the geodynamo^{6–9}.

By inverting the temporal variation of the magnetic field at the core–mantle boundary (CMB), it is possible to construct models of the flow at the top of the core¹⁰, although if no extra assumptions are made, such flow models are ambiguous. This ambiguity can be alleviated by making various dynamical assumptions, such as that the flows at the top of the core are tangentially geostrophic^{11,12}, toroidal¹³ or steady^{14,15}. From such flow models, it is possible to estimate the changes in the core angular momentum provided that

it is assumed that the part of the flow that varies with time is constant in the direction parallel to the Earth’s rotation axis (the *z*-direction)¹⁶. Doing so yields a good agreement for recent years (AD 1900–90, and especially for 1960–90) between changes in core angular momentum inferred from core flow models and those inferred from variations in the length of day (ΔLOD)^{16,17}.

One type of *z*-independent flow is that of ‘torsional oscillations’, which were suggested as an explanation for the observed decadal ΔLOD even before the discovery of the agreement between ΔLOD and core angular momentum^{18,19}. Braginsky¹⁹ noted that torsional oscillations will have a period of ~ 60 years if B_s (the component of magnetic field orthogonal to and away from the rotation axis) is ~ 0.2 mT.

Waves on decadal timescales in the core are likely to be torsional oscillations. Inertial waves have much shorter timescales (~ 1 day) whereas waves that are magnetostrophic (a balance of Lorentz, Coriolis, buoyancy and pressure forces^{20,21}) are thought to have longer timescales (~ 300 years; ref. 22). It has also been noted from models of core flow that variations of core motions in space and time are suggestive of torsional oscillations²³. The oscillations are large when compared with the variation in the overall core rotation

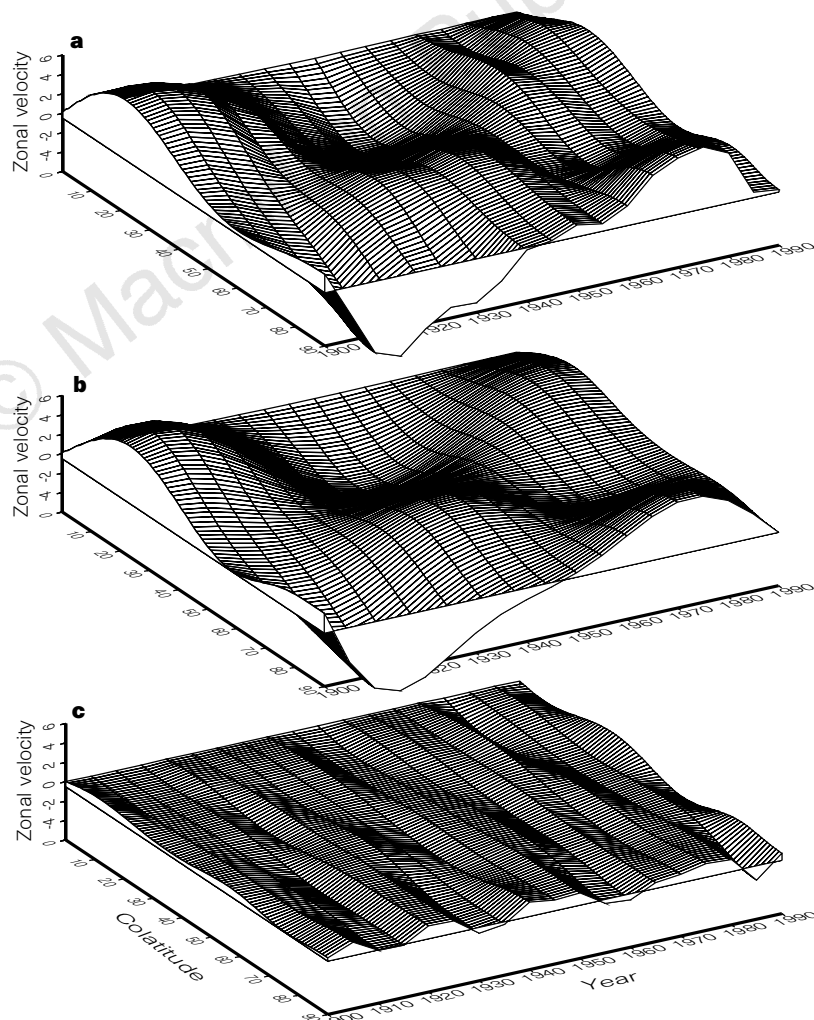


Figure 1 The axisymmetric, equatorially symmetric part of the velocity of the surface of the core (with the steady part removed) from AD 1900 to AD 1990. Velocities are given in km yr^{-1} ; colatitudes are given in degrees. **a**, Flow model estimated from the secular variation of the magnetic field and using the assumption of tangential geostrophy at the core surface to reduce the ambiguity of the

flow. The model is truncated at degree 14. Damping of spatial derivatives is applied to ensure convergence. The model is expanded in time by cubic B-splines with knots spaced at 5-year intervals. **b**, Fit of two damped harmonic waves to the model shown in **a**. The fit performed is a least-squares fit to the t^0 (*l* odd) spherical harmonic coefficients of the flow model, from AD 1900 to AD 1990. **c**, Residuals.

rate relative to the mantle reference frame, suggesting that the angular momentum within the oscillations mostly cancels^{17,23}. Thus, even though models of core flow are partly dependent on the assumptions used in their construction, given the observed correlation between core angular momentum and ΔLOD we can infer that at the very least the axisymmetric, equatorially symmetric, time-varying parts of the zonal flow are well determined.

We find that we can fit the equatorially symmetric, axisymmetric zonal velocities from a core flow model from AD 1900 to AD 1990 with a steady flow plus two damped harmonic waves, with a variance reduction of 98.85% (Fig. 1). The waveforms and frequencies of the fitted waves, which we call wave A and wave B, are shown in Fig. 2. As shown in Table 1, the real parts of the wave frequencies have a ratio close to 3:2, suggesting that the waves might be harmonics of a single wave, or generated by a common excitation mechanism. As wave-like behaviour is not assumed in the construction of the flow model, the ease with which the model may be fitted with waves adds credence to both the flow model and the presence of waves in the real core.

We can invert these waves for a model of B_s in the core and a model of 'friction' at the CMB by using the equation governing their dynamics, provided that we make the following additional assumptions: first, that the waves are torsional oscillations about a basic state; second, that this basic state is approximately steady over the

length of the study; third, that wave-basic state interactions are negligible; and last that the friction at the CMB is of the form:

$$f_\phi = F(s)u_\phi \quad (1)$$

where f_ϕ is the (real) azimuthal friction per unit area of the CMB, F is a coefficient of friction per unit area, u_ϕ is the zonal velocity of the top of the core relative to the mantle, and s is the cylindrical polar coordinate in the direction pointing away from the Earth's rotation axis. A friction of this form may represent viscous drag, topographic (pressure) coupling²⁴, or a "magnetic friction"²⁵, which may be thought of as part of the advective magnetic torque due to the field induced in a thin conducting layer at the bottom of the mantle^{26–28} by a shear between the core and mantle. $F(s)$ may also, if positive, represent net excitation. Excitation mechanisms might involve turbulent torques between the axial cylinders or the evolution of the magnetostrophic basic state of the core, which may generate unbalanced torques which lead to torsional oscillations. Over long timescales, of course, we would expect there to be as much excitation as damping of torsional oscillations. This introduces a complication, namely that the excitations of the two waves may differ.

Table 1 Parameters of fitted waves

Wave	Frequency		Period	Decay time
	Real part	Imaginary part		
A	0.0131	0.0021	76.2	74.1
B	0.0189	0.0017	52.7	95.7

The frequencies (yr^{-1}), periods (yr) and e^{-1} decay times (yr) of the fitted oscillations. (See Fig. 1 legend for details). Here, a positive imaginary part of the frequency corresponds to decay of the wave.

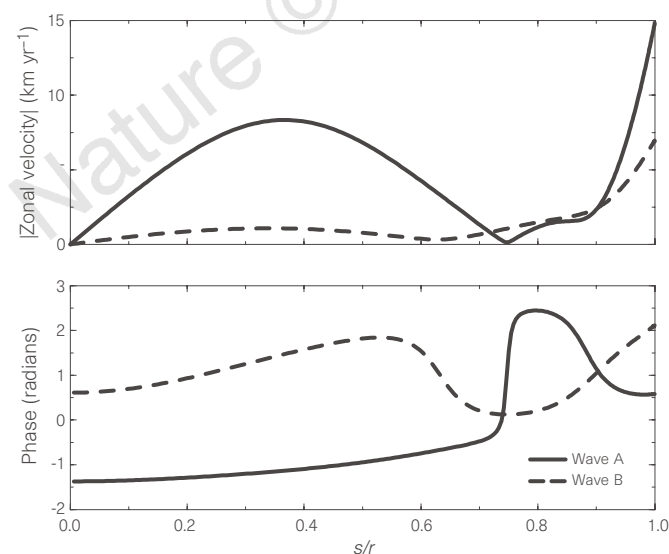


Figure 2 The amplitudes and phases of the two fitted waves (see Fig. 1 legend). r is the radius of the core, s is the distance from the Earth's axis. Wave A has a clear node at $s/r = 0.75$ where the amplitude drops to nearly zero and the phase rapidly changes by π radians. There is also a rapid change of phase around $s/r = 0.88$, which is associated with a peak of excitation in the fitted friction model. Wave B is only large near the equator, with a phase that decreases steadily from the equator to the pole, except for what may be a poorly resolved node near $s/r = 0.63$. It is not clear that wave B is well resolved, especially away from the equator. The fitted frequencies for these waves are shown in Table 1.

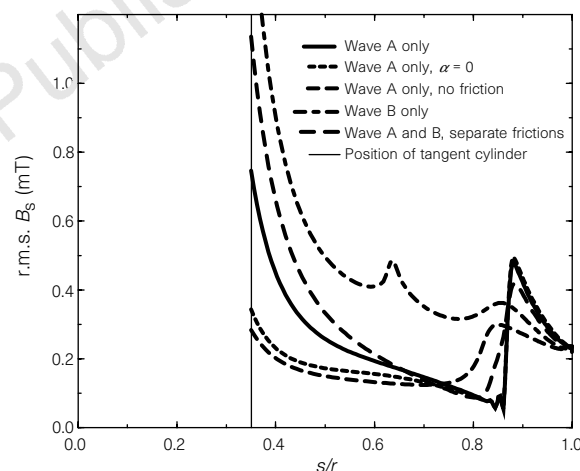


Figure 3 r.m.s. $B_s(s)$, averaged on axial cylinders, for various wave/friction model inversions. The equation that we invert is:

$$\frac{1}{2\sqrt{r^2 - s^2}} \left(\frac{M\nu(\omega + \omega_m)}{2\pi s} + \frac{1}{\mu_0\nu} \frac{\partial\omega}{\partial s} \left\{ \alpha[B_s B_z]_{z=0} + \frac{s}{\sqrt{r^2 - s^2}} [B_{s+}^2 + B_{s-}^2] \right\} \right) + \frac{i\omega r}{r^2 - s^2} F + \frac{1}{\mu_0\nu} \left(\left(\frac{\partial^2\omega}{\partial s^2} + \frac{\partial\omega}{\partial s} \left(\frac{3}{s} - \frac{s}{r^2 - s^2} \right) \right) + \frac{\partial\omega}{\partial s} \frac{\partial}{\partial s} \right) B_s^2 = 0$$

where M is the mass per unit thickness of the axial cylinder, ν is the complex frequency, $\omega = u_\phi/s$, ω_m is the change in rate of rotation of the mantle associated with the torsional oscillation, z_+ and z_- are the coordinates of the intersection of the axial cylinder with the CMB, $\{\}$ denotes zonal average, and α depends on the boundary conditions. The oscillating azimuthal magnetic field B_ϕ^s cannot diffuse through the CMB if it is toroidal and the mantle is insulating, but if B_ϕ^s has a poloidal portion or if the bottom of the mantle conducts, then some B_ϕ^s will escape. $\alpha = 0$ gives the toroidal B_ϕ^s /insulating mantle limit, $\alpha = 1$ implies full penetration of B_ϕ^s through the CMB. For the inversions shown, $\alpha = 1$ except where marked otherwise. This figure shows several extreme cases: the fits for the inversion of wave B only and the inversion with no friction were markedly inferior. Wave B is probably only poorly resolved, as its amplitude is small except near the equator (see Fig. 2). We have most confidence in the fits of wave A with friction, and in the most robust features, which are that r.m.s. $B_s \rightarrow 0.24$ mT at the equator, rises to a maximum and then drops to a minimum at $s \approx 0.8$, and then rises towards the tangent cylinder. The inversion is carried out by expanding F and B_s^2 in cubic B-splines and discretizing the above equation. Choice of reasonable error model makes negligible difference to the inversion. For the inversion of wave A, there is some numerical instability close to the minimum: this is reduced as the number of splines is increased, and does not affect the fit away from this region.

With these assumptions, torsional oscillations obey a complex, first-order differential equation that is linear in F and $\overline{B_s^2}$. As we know the form of the torsional oscillations, we can invert the real and complex parts of the equation for a model of F and $\overline{B_s^2}$ as a function of s .

As we neglect the dynamics of inner-core coupling in this study, we can only invert for cylinders between the inner core tangent cylinder (the axial cylinder that is tangential to the solid inner core) and the equator. Figure 3 shows models of root-mean-square B_s averaged on axial cylinders (henceforth r.m.s. B_s) from various inversions involving wave A, wave B or both. The magnitude of r.m.s. B_s is of the same order as that of the radial magnetic field at the surface of the core (~ 0.4 mT)—they should be, and are, identical at the equator.

The radial magnetic field at the CMB²⁹ (B_{rCMB}) shows several patches of strong field and of reversed field. In particular, there is a patch of reversed flux at the north pole. This must be related to a knot of reversed magnetic field within the core. In Fig. 4 we show schematically a possible cross-section of the non-zonal magnetic field in the core. The knot of reversed flux close to the north pole might explain why r.m.s. B_s increases towards the tangent cylinder in Fig. 3. Alternatively, it is possible that some of the effects of the inner core persist even when the tangent cylinder is excluded from the inversion.

Considerable progress has been made recently in fully dynamically self-consistent numerical modelling of the geodynamo^{6–9}. Models that produce fairly similar, basically Earth-like fields at the core surface have very different internal structures. Our results may be useful for testing dynamo simulations, albeit with the caveat that this study only gives a 'snapshot' of the geodynamo, and it is possible that the internal core field evolves significantly with time even when the core is not close to a magnetic polarity reversal.

One possible reason for the difference between numerical

dynamo models is that they may be in different dynamic states. There are several alternatives for the dynamic state of the core. Two possibilities which are often considered are the 'quasi-Taylor state' where the axial magnetic torque integrated over axial cylinders cancels (Taylor's constraint) except for the part involved in torsional oscillations which is balanced by inertia, and the 'model-Z' state where the axial magnetic torque on axial cylinders in the mainstream of the flow is balanced by torques due to core–mantle coupling, and where the poloidal magnetic field tends to be nearly aligned with the z -axis (that is, B_s is small)^{3,25,30}. There are other possibilities for breaking Taylor's constraint, including turbulent or convective angular-momentum transfer in the core or non-frictional core–mantle coupling (such as that due to leakage of toroidal field into the mantle). We find relatively large B_s ($|B_s| \approx |B_{rCMB}|$), which is discouraging for the model-Z hypothesis. However, it is still possible for the core to be in a model-Z state if core–mantle coupling is large enough. We should be able to check whether the torques implied by a model-Z-type dynamo are consistent with our model of r.m.s. B_s and F .

We note that our friction models show significant lateral variation, which may be due to the excitation but which otherwise might indicate lateral variation in properties such as conductivity or topography at the bottom of the mantle. □

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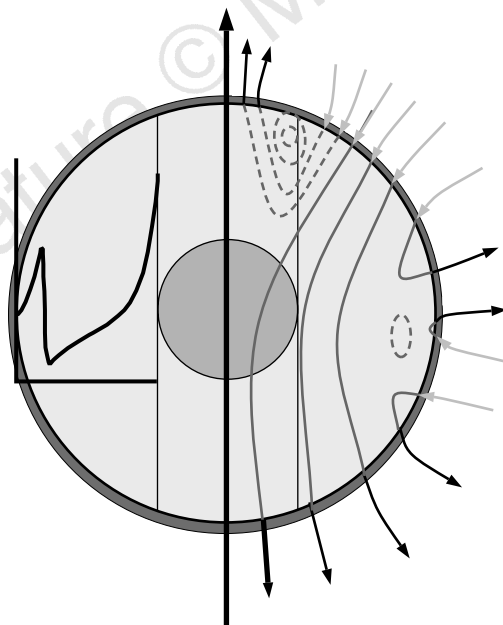


Figure 4 Cartoon of a possible cross-section of poloidal magnetic field through the core. The central circle represents the inner core, and the vertical lines mark the border of the tangent cylinder. Outside the core, black lines mark field leaving the core, grey lines mark field entering the core. Inside the core, dashed lines mark clockwise-circulating field, solid lines mark anticlockwise-circulating field. In a three-dimensional core, knots might also lie in the plane perpendicular to this cross section. For illustrative purposes, on the left hand side is a graph of r.m.s. B_s (wave-A-only inversion) as a function of distance from the rotation axis.

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Self-similarity of extinction statistics in the fossil record

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The dynamical processes underlying evolution over geological timescales remain unclear^{1,2}. Analyses of time series of the fossil record have highlighted the possible signature of periodicity in mass extinctions^{3,4}, perhaps owing to external influences such as meteorite impacts. More recently the fluctuations in the evolutionary record have been proposed to result from intrinsic nonlinear dynamics for which self-organized criticality provides an appropriate theoretical framework^{5–7}. A consequence of this controversial⁸ conjecture is that the fluctuations should be self-similar, exhibiting scaling behaviour like that seen in other biological⁹ and socioeconomic^{10,11} systems. The self-similar character is described by a $1/f$ power spectrum $P(f)$, which measures the contributions of each frequency f to the overall time series. If self-similarity is present, then $P(f) \approx f^{-\beta}$ with $0 < \beta < 2$. This idea has not been sufficiently tested, however, owing to a lack of adequate data. Here we explore the statistical fluctuation structure of several time series obtained from available palaeontological data bases, particularly the new 'Fossil Record 2'¹⁸. We find that these data indeed show self-similar fluctuations characterized by a $1/f$ spectrum. These findings support the idea that a nonlinear response of the biosphere to perturbations provides the main mechanism for the distribution of extinction events.

The Phanerozoic record of life consists almost entirely of extinct groups of organisms, indicating that extinction is the fate of most lineages and that no biotas are infinitely resilient¹³. Considerable effort has been devoted to explaining the so-called mass extinction events in terms of external physical events^{14,15}. The dynamics of the biosphere are reflected, to some extent, in the time series provided by the fossil record. Different theories have been tested by means of analyses of this data set.

A direct inspection of the available time series¹⁶ of fluctuations in the number of Ammonoidea families (Fig. 1) suggests a fractal structure in this fossil record, which is consistent with statistical measures such as the frequency distribution of extinctions and

lifetimes^{12,17}, which also exhibit power-law decay. Although self-organized criticality (SOC) is not the only mechanism leading to power laws, we should first test whether or not there is evidence for $1/f$ dynamics in real time series. We have tested this idea by using the data sets available from 'The Fossil Record 2' which includes all groups that have a fossil record¹⁸ as well as information from other databases, such as the Sepkoski compilation of Phanerozoic diversity and extinction events for marine genera¹⁹.

Using a given time series $\xi(t)$ ($t = 1, 2, \dots, N$), where the scale is typically in millions of years, we compute $P(f)$; this is done by using the discrete Fourier transform²⁰ through a standard

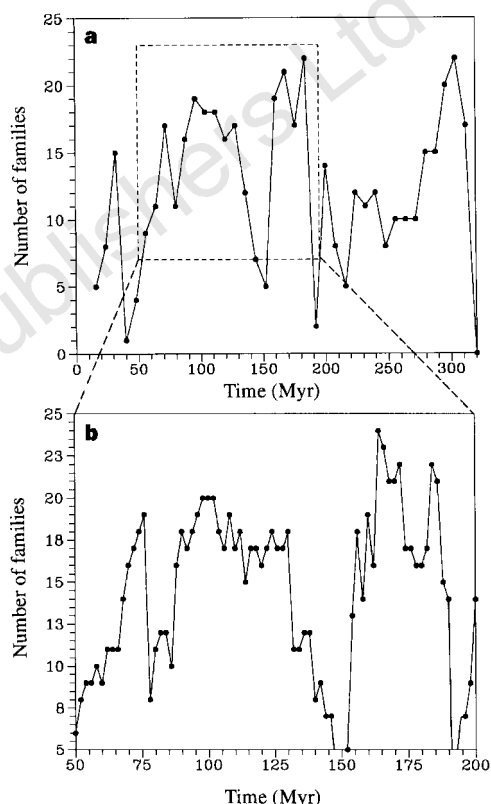


Figure 1 Self-similarity in the fossil record time series (redrawn from House¹⁶). Here we show the fluctuations in families of Ammonoidea (over a period of 320 Myr, with a time resolution of 2 Myr). In **a** only one of each four original data points is shown, giving a coarse resolution of 8 Myr. In **b** we plot a segment of the previous data, but at full (2-Myr) resolution.

Table 1 Scaling exponents for family origination and extinction

	Origination	Total ext.	Per cent ext.	Tot ext. rate	PF ext. rate
Global (min.)	0.94 ± 0.01	0.93 ± 0.01	0.97 ± 0.01	0.83 ± 0.02	0.87 ± 0.02
	0.92 ± 0.03	0.98 ± 0.01	1.00 ± 0.01	0.86 ± 0.02	0.87 ± 0.02
Global (max.)	0.93 ± 0.01	0.92 ± 0.02	0.97 ± 0.01	0.84 ± 0.02	0.88 ± 0.02
	0.91 ± 0.03	0.93 ± 0.01	0.99 ± 0.01	0.88 ± 0.02	0.90 ± 0.02
Terr. (min.)	0.92 ± 0.01	0.92 ± 0.01	0.95 ± 0.01	0.82 ± 0.01	0.88 ± 0.01
	0.90 ± 0.02	0.95 ± 0.01	0.98 ± 0.01	0.91 ± 0.02	0.94 ± 0.01
Terr. (max.)	0.91 ± 0.01	0.89 ± 0.02	0.92 ± 0.02	0.81 ± 0.03	0.87 ± 0.02
	0.90 ± 0.02	0.91 ± 0.01	0.95 ± 0.01	0.87 ± 0.03	0.90 ± 0.02
Marine (min.)	0.97 ± 0.01	0.95 ± 0.01	0.98 ± 0.01	0.88 ± 0.02	0.88 ± 0.02
	0.99 ± 0.01	1.00 ± 0.01	1.00 ± 0.01	0.87 ± 0.02	0.89 ± 0.02
Marine (max.)	0.93 ± 0.01	0.91 ± 0.01	0.97 ± 0.01	0.91 ± 0.02	0.92 ± 0.02
	0.98 ± 0.01	0.96 ± 0.02	1.01 ± 0.01	0.89 ± 0.02	0.90 ± 0.02

Exponents are given for different extinction metrics, involving global, terrestrial and marine time series². Both the exponent for the power spectrum β (first row of each pair of rows) and the r.m.s. exponent α (second row) are given. 'Origination' indicates total number of new families; 'Total ext.' indicates total number of extinct families; 'Per cent ext.' indicates per cent of extinction, measured relative to the number of families in existence; 'Tot. ext. rate' (TER) indicates total extinction rate; 'PF ext. rate' indicates per family extinction rate, measured as the TER divided by taxic diversity. Deviations from the $\beta = 1$ exponent are more frequent when rate-dependent measures are considered. This is expected as more information is involved and deviations from sampling errors are more likely to occur.

fast-Fourier-transform algorithm (FFT). $P(f)$ is

$$P(f) = \frac{1}{\sqrt{N}} \sum_{\tau=1}^N C(\tau) \exp\left(\frac{-i2\pi f\tau}{N}\right) \quad (1)$$

where $C(\tau) = \langle \xi(t)\xi(t+\tau) \rangle$ is the so-called autocorrelation function. Then a log-log plot of $P(f)$ on f is used (Fig. 2) and the scaling exponent β is computed from a least-squares fit (a linear regression of log-transformed data is better than nonlinear fit on raw data because the residual error will be distributed as a quadratic and the minimum error is guaranteed). If long-range correlations are present, then a scaling $C(\tau) \approx \tau^{-\gamma}$ will be observed with $\gamma = 1 - \beta$ (refs 10, 21).

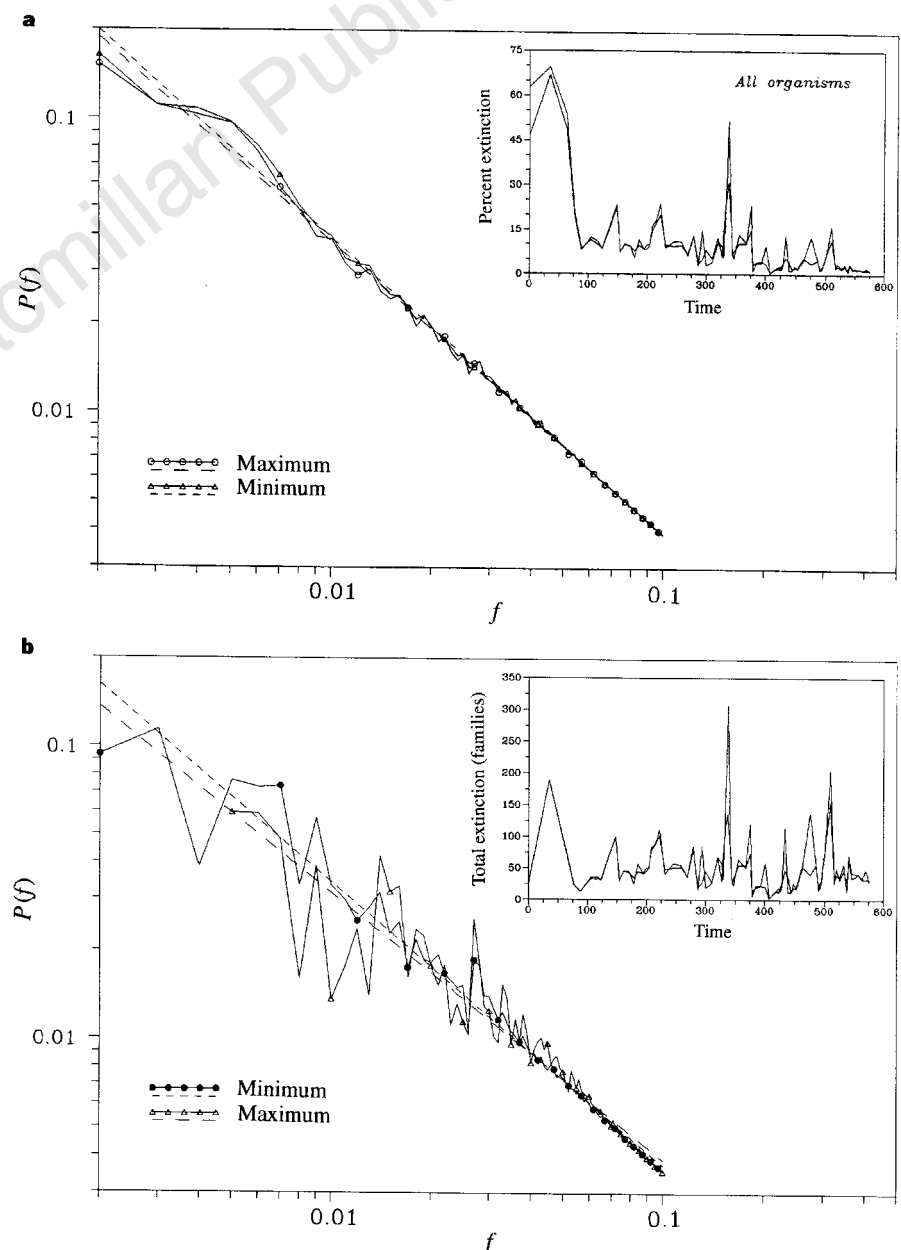
Several time series have been used, involving both origination and different measures of extinction for families. First, we consider the extinction sizes in families. Here the available data sets are mainly for stratigraphic stages, which have an average duration of 7 Myr (ref. 18). So in order to get an equally spaced data set with 1-Myr resolution we need to generate more points. We have used a direct, linear interpolation, the results of which are shown in Table 1. Because this method can lead to spurious correlations, a different

procedure was also used to test the robustness of our results: a step-function approach to each stage is introduced, together with a square-root transformation^{20,21}. Both methods gave basically the same results. Both minimum and maximum measures are available, based on assessments of uncertainty about dates of origin and extinction and other factors^{2,22}. In Table 1 we show our results for family origination and extinction by using four different extinction metrics²: most of the obtained scaling exponents are close to $\beta \approx 1$. There is some drift towards lower β for time series based on rates of extinction. This could be due to the biases in the estimation of the total number of families per stage². A decrease in β is expected as a consequence of errors introduced by estimated stage duration. These durations are poorly constrained, especially for stages before 100 Myr ago, and their estimates vary considerably among available geological timescales.

An additional, complementary measure is obtained by means of the use of 'random walk' methods able to detect long-range correlations^{10,21,23-25}. Starting from the time series $\xi(t)$, the running sum

$$y(\tau) \equiv \sum_{i=t}^{\tau} \xi(i) \quad (2)$$

Figure 2 a, Pattern of family extinctions through time of all organisms in terms of the number of families. Maximum and minimum curves² are shown (inset) as well as the corresponding power spectra (main figure). **b**, Fluctuation in the marine families (inset) and the corresponding power spectrum (main figure). Both series are in a timescale of Myr and in both cases the spectrum is not like white, uncorrelated noise ($\beta = 0$) but almost like $1/f$ -spectra, characteristic of long-range correlations in scale-invariant systems.



is computed, and the root-mean-square (r.m.s.) fluctuation $F(\tau)$ is obtained from

$$F(\tau) \equiv [(\langle \delta y(\tau)^2 \rangle) - (\langle \delta y(\tau) \rangle)^2]^{1/2} \quad (3)$$

where $\delta y(\tau) = y(t_0 + \tau) - y(t_0)$. The angle brackets indicate an average over all times t_0 . If $\{\xi(t)\}$ is random or a simple Markov process, then we have $F(t) \approx t^{1/2}$. But if no characteristic timescale is involved, then a scaling $F(t) \approx t^\alpha$ is observed with $\alpha \neq 1/2$. The r.m.s. exponent and β are related through $2\alpha = \beta + 1$. We have computed α for all the previous data sets, and the resulting exponents are given in Table 1 (see also Fig. 3). We can see that α is also close to one and that all the pairs (α, β) are consistent with the previous relation.

We also analysed the extinction pattern for other taxonomic levels. The study of the total extinction rate for genera of marine animals¹⁹ gave $\beta = -0.86 \pm 0.03$ and $\alpha = 0.91 \pm 0.03$ using the proportional rate of extinction and $\beta = -0.83 \pm 0.02$, $\alpha = 0.89 \pm 0.03$ for the absolute rate of extinction. We can extend our analysis to other types of data, involving different particular groups of organisms. We have used two particularly well known data sets: fluctuations in Ammonoidea families¹⁶ and the number of planktic foraminiferal species from the Jurassic period to the Holocene epoch²⁶. Here no interpolation is needed and again well defined power laws were obtained: $\beta = -0.88 \pm 0.03$ and $\alpha = 0.93 \pm 0.04$ for Ammonoidea and $\beta = -0.86 \pm 0.03$ and $\alpha = 0.91 \pm 0.03$ for Foraminifera.

These results are consistent with a nonlinear, self-similar behaviour of the biosphere. Where do these scale-free dynamics come from? There are several possibilities; one of them is SOC, where fluctuations over all timescales and power laws are a direct consequence of criticality⁵⁻⁷. Some authors have shown that power laws do not need to be generated through a SOC phenomenon¹⁷ but can result from stresses (either biotic or abiotic) to which species are subjected by their environment. Additionally, the reported fractal nature of taxonomic systems²⁷ shows that behind the self-similar time series, a fractal-like organization of the biosphere is also present. Recent SOC models of macroevolution have shown that these scaling relations naturally fit into a self-organized, nonlinear generic process^{13,23}. Our results are also in agreement with other studies of palaeontological time series based on other methods from nonlinear dynamics^{7,28} as forecasting techniques. In all these studies, data analysis supports the internal biotic organization as the basic component for the response of the biosphere to external

perturbations.

Furthermore, these analyses provide evidence that might help resolve two debates about macroevolutionary dynamics. The first is the nature of mass extinctions: are they qualitatively, or merely quantitatively, different from normal (background) levels of extinction? Early statistical tests²⁹ suggested that there was a clear distinction, and that the five largest (the 'big five'; ref. 14) mass extinctions were qualitatively distinct. This view was supported by the finding that times of mass extinction were associated with selective regimes that were utterly different, and unpredictable, from observations during background times¹³. Comparisons of probabilities and magnitudes of extinction events have subsequently suggested³ however, that the 'big five' could be the skewed end of a continuous distribution of extinction events of different intensity. This view is supported by the scale-free pattern revealed by our study.

The second debate that is addressed by our study concerns the causes of extinctions. When Raup and Sepkoski⁴ proposed that mass extinction events over the past 250 Myr had followed a periodic pattern of occurrence every 26 Myr, many workers focused on extraterrestrial causes. Raup³ has extended this model to postulate that all extinction events can be explained by extraterrestrial impacts, the magnitude of the event depending broadly on the magnitude of the object striking the Earth (see also Newman¹⁷). A different (but perhaps complementary) view⁵ is that extinction events are triggered by a multiplicity of factors, and that the internal dynamics of the system play an important role. Although the role of nonlinear dynamics in extinction and diversity is not new in palaeobiology^{1,26,30,31}, previous analysis dealing with fluctuations in the fossil record involved low-dimensional, deterministic approaches based on chaotic dynamics. But it seems clear that the underlying assumptions (such as the low-dimensional character of the dynamics, the lack of stochastic effects or the constancy of parameters) are unlikely to hold. A high-dimensional picture where many species evolve and interact seems more consistent with the process of macroevolution, although the specific mechanisms that could drive the biosphere dynamics are under discussion³². Our studies, consistent with scale-free pattern of the fossil record, support this latter view. Future analyses could usefully explore factors such as the stationarity of the previous data sets or the Signor-Lipps effect (the 'backward smearing' of extinctions resulting from incomplete sampling³³) and their importance in generating correlations. □

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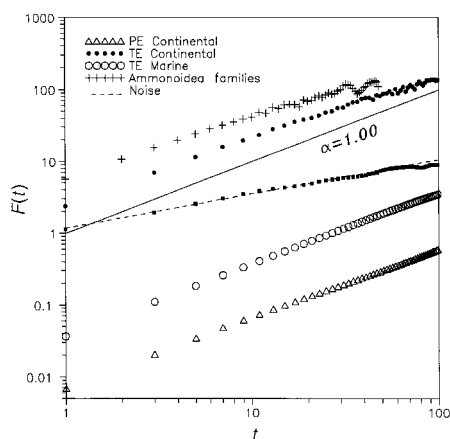


Figure 3 Log-log plot of the root-mean-square $F(t)$ for different (typical) palaeontological time series (see equations (2) and (3) in the text). Here both per cent extinction (PE) and total extinction (TE) for different data sets have been used. We also show the scaling for fluctuations in families of Ammonoidea and the corresponding scaling for a random white-noise process from a $N = 150$ time series. Here $\alpha = 1/2$.

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Genetic tagging of humpback whales

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The ability to recognize individual animals has substantially increased our knowledge of the biology and behaviour of many taxa¹. However, not all species lend themselves to this approach, either because of insufficient phenotypic variation or because tag attachment is not feasible. The use of genetic markers ('tags') represents a viable alternative to traditional methods of individual recognition, as they are permanent and exist in all individuals. We tested the use of genetic markers as the primary means of identifying individuals in a study of humpback whales in the North Atlantic Ocean. Analysis of six microsatellite loci^{2,3} among 3,060 skin samples collected throughout this ocean allowed the unequivocal identification of individuals. Analysis of 692 'recaptures', identified by their genotype, revealed individual local and migratory movements of up to 10,000 km, limited exchange among summer feeding grounds, and mixing in winter breeding areas, and also allowed the first estimates of animal abundance

based solely on genotypic data. Our study demonstrates that genetic tagging is not only feasible, but generates data (for example, on sex) that can be valuable when interpreting the results of tagging experiments.

Skin biopsy⁴ or sloughed skin⁵ samples from free-ranging humpback whales (*Megaptera novaeangliae*) were collected across the North Atlantic between 1988 and 1995. Total-cell DNA was extracted⁶, and the sex⁷ and genotype at six mendelian inherited⁸ microsatellite loci⁹ were determined for each sample. From the 3,060 samples analysed, we detected 2,368 unique genotypes. The expected number of samples collected from different individuals with identical genotype arising by chance was estimated at less than one (see Methods). Because of this, and the fact that all samples with identical genotypes were of consistent sex, we believe that the 3,060 samples represented 2,368 individual whales. Of the 692 recaptures observed during the study, 216 occurred on the summer feeding grounds (Fig. 1). Of these, 96% ($n = 207$) occurred within the same feeding area (Fig. 1), confirming previous behavioural^{10,11} and genetic^{12,13} observations of maternally directed fidelity to specific feeding grounds. The remaining 4% ($n = 9$) of the recaptures on the summer feeding grounds were detected in different but adjacent feeding grounds (Fig. 1). However, significantly more recaptures were detected within these sampling areas than would be expected if the areas constituted one intermixing feeding aggregation and is consistent with the notion of maternally directed site fidelity (see Methods).

Of the 114 individuals recorded on both summer feeding and winter breeding grounds (Fig. 1), two had migrated from the West Indies to either Jan Mayen or Bear Island (in the Barents Sea). These genetic recaptures represent the most extensive one-way movements (6,435 and 7,940 km, respectively) recorded in this study, and support recent findings^{13,14} that whales feeding in the Barents Sea share a common breeding ground with other North Atlantic humpback whales. In three other individuals, which were each sampled on three occasions, movements were documented from a feeding ground to the breeding range and back, involving minimum migration distances of up to 10,000 km between the first and last sampling event. No feeding ground was disproportionately represented among the recaptures in the West Indies (Fig. 1), supporting the current view that humpback whales in the North Atlantic constitute a single panmictic population^{13,15,16} (G -test, $G_{\text{d.f.}} = 4.68$, $P < 0.46$).

As with traditional identification methods¹, microsatellite data lend themselves to abundance estimation using mark-recapture statistical methods¹⁷, although to our knowledge this has not previously been attempted. Using breeding-ground samples collected during 1992 and 1993, we estimated the North Atlantic humpback whale population at 4,894 (95% confidence interval, 3,374–7,123) males and 2,804 (95% confidence interval, 1,776–4,463) females. This total of 7,698 whales is substantially (albeit not significantly) higher than the most recent previous photographic-based estimate of 5,505 (ref. 10) (95% confidence interval, 2,888–8,122). Preliminary results from new and more reliable photographic estimates are also larger than previous estimates (T.S. *et al.*, manuscript in preparation), and could partly be due to population growth during the intervening decade since the previous estimate¹⁸. The significantly different estimates for males and females are unexpected given the even sex ratio observed on the feeding grounds¹⁹ (Table 1) and among 198 calves that we sampled in the breeding range (data not shown). The estimates are independent of between-sex sampling biases, and so the observed deficit of females probably reflects within-sex behavioural differences, for example that individual females display a higher degree of preferences with respect to region and/or residence time in the breeding range than do males.

Our results demonstrate that genetic tagging is effective even in a large population of wide-ranging and inaccessible mammals such as cetaceans. Further, the data obtained from genetic tags can be used to address evolutionary²⁰, demographic¹⁹ and behavioural²¹

questions to which traditional tagging methods are unsuited. Because all eukaryotes possess microsatellites²², individuals within any taxon can in principle be identified reliably from minute quantities of tissue. Such tissue is commonly derived from biopsies²³, but can also come from sloughed skin⁵, shed hair²⁴ or fecal material^{25,26}, thus potentially allowing genotyping and individual recognition even of unobserved animals. However, the validity of a genetic tag depends on there being a sufficiently low probability of identity²⁷. An underestimate of this probability can be caused by unrecognized population substructure or linkage disequilibrium among loci. Additional analyses addressing these issues, as well as checks with other data (such as the sex of recaptures, as in this study) must be performed to ensure the validity of the overall probability of identity. Similarly, a genetic tag consists of data from multiple loci, each of which are prone to handling errors in the laboratory. With proper laboratory procedures, such errors can be rendered minimal (here estimated to 0.0011 per locus), and so do not represent a serious obstacle to the detection of recaptures. Recaptures with an erroneous genotype will almost certainly be among the samples that match at all but one locus. If a sufficient number of loci has been analysed, none or few samples are expected to match at all but one locus, and so re-analysis of the discrepant locus presents only a minor additional effort. □

Methods

Expected number of samples with identical genotypes. The number of samples from different individuals with identical genotypes (across all loci) arising by chance was estimated from the probability of identity (I)²⁷. No difference in the estimate was observed whether I was estimated from

all samples of unique genotypes only. The expected number of samples from different individuals with identical genotypes arising by chance was first estimated separately within each sampling area, and subsequently between sampling areas (after removal of duplicate genotypes within each sampling area). The expected total number of such matches was estimated to be 0.32 and 0.27 within and between areas, respectively. No significant degree of linkage disequilibrium (which could cause an underestimate of I), nor any significant deviations from the expected Hardy–Weinberg proportions of genotypes, was observed after the removal of duplicate genotypes.

Expected number of matches between the Gulf of St Lawrence and Newfoundland/Labrador. The probability of observing six recaptures between years in the Gulf of St Lawrence was assessed by Monte Carlo simulations, under the assumption that the Gulf of St Lawrence and Newfoundland/Labrador constitute one intermixing feeding aggregation. Several tests, each of 1,000 simulations, were conducted over a range of the most likely abundance estimates derived from the data. Each simulation was conditioned on the number of recaptures observed in the sample and the order of sampling in the two areas. The probability of observing six or more individuals sampled more than once in the Gulf of St Lawrence (if part of the same feeding ground as Newfoundland/Labrador) was estimated to less than 0.0001.

Estimation of abundance and log-likelihood ratio test of equal numbers of males and females on the breeding range. Estimates of abundance and 95% confidence intervals were calculated as suggested previously¹⁷. In 1992, 382 males and 231 females were sampled on the breeding range; the corresponding numbers for 1993 were 408 and 265. Between the two years we observed 31 and 21 recaptures of males and females, respectively. The statistical significance of the difference in the estimated number of males and females was assessed with a likelihood-ratio test of the null hypothesis that the number of males was equal

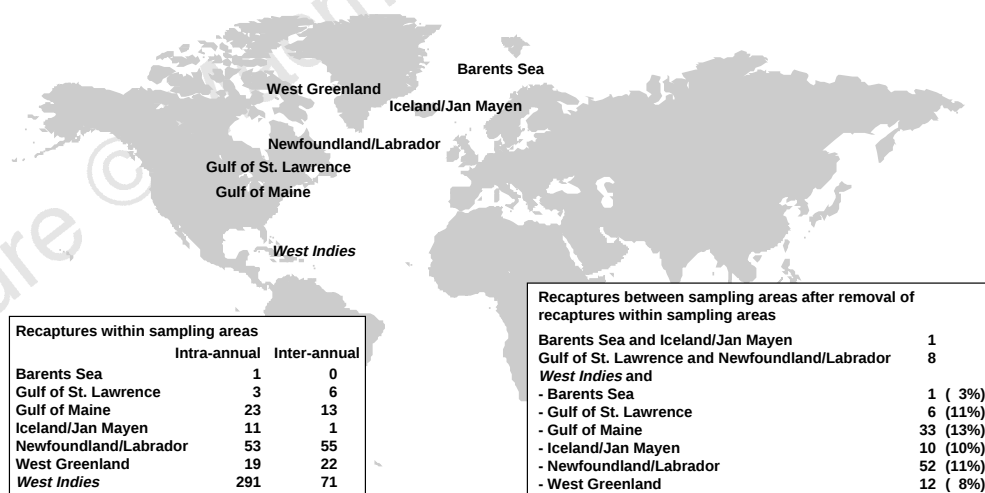


Figure 1 Numbers and distribution of recaptures. Sampling areas in italics are in the breeding range. The percentage in parentheses denotes the proportion of recaptures relative to the total number of genotypes tagged in the given sampling area.

Table 1 Sample sizes, probabilities of identity, number of genotypes and individuals of each sex

Sampling area	Period	Samples	I ($\times 10^{-7}$)*	95% confidence limits ($\times 10^{-7}$)†	Genotypes	Males	Females
Barents Sea	1992–1993	36	8.46	4.9–38	35	13	22
Gulf of St Lawrence	1990–1995	65	1.94	1.26–5.52	56	28	28
Gulf of Maine	1990–1995	292	1.38	1.02–2.11	256	118	138
Iceland/Jan Mayen	1991–1993	112	1.43	0.88–3.28	100	50	50
Newfoundland/Labrador	1991–1995	572	1.34	1.07–1.86	464	237	227
West Greenland	1988–1994	189	1.23	0.89–2.10	148	75‡	72‡
West Indies (breeding range)	1989–1995	1,794	1.81	1.57–2.15	1,432	884‡	545‡
Without intra-area recaptures		2,491	1.51	1.34–1.75	2,368		
Unique genotypes only		2,368	1.51	1.32–1.72		1,331‡	1,033‡

* Probability of identical genotype²⁷ across all loci calculated from all samples (including recaptures).

† Estimated from 1,000 bootstrap samples.

‡ No sex was obtained for four samples.

to the number of females, assuming that the number of recaptures is hypergeometrically distributed. Asymptotically the test statistic ($-2 \ln(\lambda)$, where λ is the likelihood ratio) is chi-square distributed with one degree of freedom, which implies that the probability of $-2 \ln(\lambda)$ exceeding 4.14 is 0.042. Monte Carlo simulations (10,000 replicates) confirmed this probability.

Estimation of error rate. The error rate was estimated from the samples with identical genotypes at five but not six loci. From 2,368 genotypes just 64 such pairs (in all 117 unique genotypes) were detected, in 19 of which the individual from which the sample was collected had been identified by its natural markings²⁸. Of these 19 incidences just 3 were from the same individual. Hence the 117 samples were estimated to include 9 samples with an incorrect genotype, which equals an error rate of 0.0011 per locus after inclusion of the 2 times 692 samples which match on all loci (presumably determined correctly). The upper limit of the 95% confidence interval was estimated to 0.0027 from Monte Carlo simulations, assuming a binomial distribution of errors, and a hypergeometric distribution of photographed whales.

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Neurodegeneration in Lurcher mice caused by mutation in $\delta 2$ glutamate receptor gene

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Lurcher (*Lc*) is a spontaneous, semidominant mouse neurological mutation¹. Heterozygous Lurcher mice (*Lc*+) display ataxia as a result of a selective, cell-autonomous and apoptotic death of cerebellar Purkinje cells during postnatal development^{2–4}. Homozygous Lurcher mice (*Lc/Lc*) die shortly after birth because of a massive loss of mid- and hindbrain neurons during late embryogenesis⁵. We have used positional cloning to identify the mutations responsible for neurodegeneration in two independent *Lc* alleles as G-to-A transitions that change a highly conserved alanine to a threonine residue in transmembrane domain III of the mouse $\delta 2$ glutamate receptor gene (*GluR δ 2*). *Lc*+ Purkinje cells have a very high membrane conductance and a depolarized resting potential, indicating the presence of a large, constitutive inward current. Expression of the mutant *GluR δ 2^{Lc}* protein in *Xenopus* oocytes confirmed these results, demonstrating that *Lc* is inherited as a neurodegenerative disorder resulting from a gain-of-function mutation in a glutamate receptor gene. Thus the activation of apoptotic neuronal death in Lurcher mice may provide a physiologically relevant model for excitotoxic cell death.

The excitatory actions of glutamate in the central nervous system are mediated by several classes of glutamate receptors. Ionotropic glutamate receptors are ligand-gated cation channels that can be divided by their pharmacological properties into two classes: the NMDA (*N*-methyl-D-aspartate) and the AMPA (α -amino-3-hydroxy-5-methyl-4-isoxazolepropionate)/kainate receptor types⁶. By sequence comparison, *GluR δ 2* is equally distantly related ($\sim 25\%$ amino-acid identity) to both of these glutamate receptor subfamilies. It does not display glutamate binding or ion-channel activity when injected into *Xenopus* oocytes or expressed in mammalian cells either alone or in combination with other glutamate receptor subunits^{7,8}. However, *GluR δ 2* null mutant mice lack cerebellar long term depression⁹, a form of plasticity at the parallel fibre–Purkinje cell synapse that is thought to be important for some forms of motor learning.

We have designed an inter-subspecific backcross between *Lc* and CAST/Ei mice and established a detailed genetic and physical map over a three-megabase region of mouse chromosome 6 (Fig. 1)^{10,11}. From the 504 informative meioses, four recombination events define the *Lc* locus, of which three are on the telomeric side and one on the centromeric side. The non-recombinant region identified by these recombination events is approximately 110 kilobases in length, and a physical contig consisting of four yeast artificial chromosomes (YACs) and five bacterial artificial chromosomes (BACs) covering this locus has been established^{10,11}. A second *Lc* allele (*Lc^J*), which is phenotypically indistinguishable from *Lc*, has

recently been found in an inbred genetic background (BALB/cByJ)¹².

Analyses of the 110-kb *Lc* region resulted in the identification of three exons (A, B and C) from the mouse *GluRδ2* gene (Fig. 1). This gene covers a genomic segment of ~800 kb that contains the entire 110-kb non-recombinant *Lc* region. Using primers within exon B, single-strand conformation polymorphism (SSCP) assays on reverse transcription–polymerase chain reaction (RT–PCR) products revealed identical *Lc*-specific bands from *Lc* and *Lc*^l (data not shown). These results were confirmed using intron primers flanking exon B to perform SSCP analysis on genomic DNA. A single SSCP was shared by *Lc* and *Lc*^l mice, but was completely absent from all five possible inbred parental strains (DBA, C57B6, C3H and CBA for *Lc* (ref. 10), and BALB/cByJ for *Lc*^l (ref. 12) and from the divergent strain (CAST/Ei) used in all genetic crosses (Fig. 2a). Two additional sets of intron primers flanking exon B also revealed this *Lc*-specific SSCP (data not shown), but similar analyses performed on the other two exons (A and C) failed to display abnormal, *Lc*-specific bands (data not shown).

To identify the *Lc* mutations, sequence analysis of these genomic and RT–PCR products were performed. These data demonstrated an identical G-to-A transition at nucleotide position 1,960 in exon B that is specific to both the *Lc* and *Lc*^l mutants (Fig. 2a). This change is found in the *Lc*^l allele but not in its parental strain (Balb/cByJ), demonstrating that this is the mutation responsible for the *Lc* phenotype, as this allele arose only about three years ago in a completely inbred genetic background¹². This mutation results in replacement of a non-polar alanine residue with a polar threonine residue at amino-acid position 654 (A654T) in the third putative transmembrane domain (TMIII) of *GluRδ2* (refs 6, 7). This residue is present in a highly conserved domain of nine amino acids that is shared by members of the ionotropic glutamate receptor family (Fig. 2b). These data lead us to conclude that the *Lc* phenotype

results from this mutation in the *GluRδ2* gene.

To investigate the physiological effects of the *Lc* mutation, Purkinje cells in thin slices of cerebellar vermis from postnatal-day-10 and -11 mutant and wild-type mice were analysed electrophysiologically. Although *Lc*^l Purkinje cells were less abundant and appeared less healthy than their wild-type counterparts, *Lc*^l Purkinje cell somata sealed readily to patch pipettes, and could be held in the whole-cell recording mode for up to an hour. Excitatory postsynaptic currents evoked by stimulating parallel fibre axons in the molecular layer were superficially normal, as were spontaneous inhibitory postsynaptic currents (data not shown). However, *Lc*^l Purkinje cells exhibited a dramatic and severe physiological phenotype. The magnitude of the holding current required to clamp the neuron at -70 mV was far greater for *Lc*^l than for wild-type Purkinje cells (Fig. 3a). Initial holding current was -890 ± 191 pA in *Lc*^l (mean ± s.e.m., *n* = 7) and -150 ± 12 pA in wild-type Purkinje cells (*n* = 10; *P* = 0.012 by two-tailed heteroscedastic *t*-test). In addition, initial membrane conductance was much larger in *Lc*^l (27.8 ± 6.8 nS, *n* = 7) than in wild-type Purkinje cells (5.4 ± 0.5 nS, *n* = 9, *P* = 0.022). Finally, resting potential was depolarized in *Lc*^l (-34.8 ± 2.1 mV, *n* = 4) relative to wild-type (-54.3 ± 1.4 mV, *n* = 7, *P* = 0.000034).

These data strongly suggest the presence of a large, constitutively active conductance in *Lc*^l Purkinje cells, which gives rise to a constitutively active inward current. This increase in conductance could result from a specific effect such as an alteration in the properties of ion channels, or a nonspecific effect such as damage to the integrity of the cell membrane or the membrane–pipette interface. To distinguish between these alternatives, we substituted *N*-methyl-D-glucamine (NMDG), a relatively large organic cation, for most Na⁺ in the external saline, and found a profound decrease in the magnitude of the holding currents of *Lc*^l Purkinje cells (Fig. 3b, c). The average change in holding current in *Lc*^l Purkinje

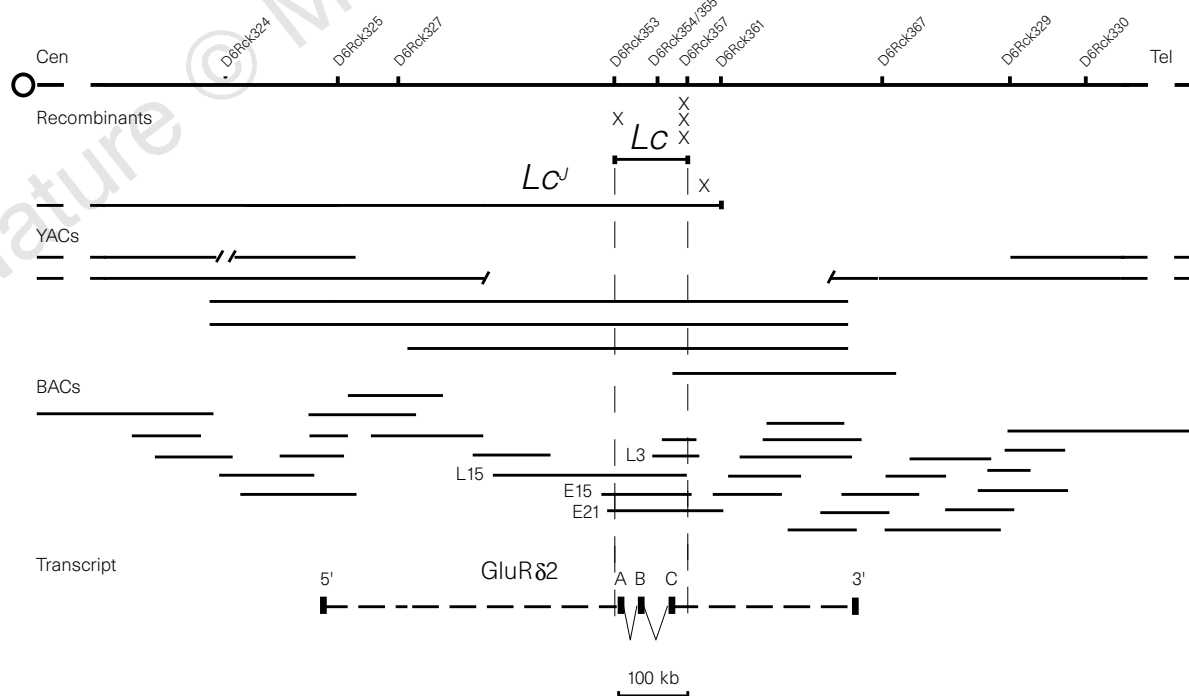


Figure 1 Physical and genetic map of the Lurcher locus and the *GluRδ2* gene on mouse chromosome 6 (refs 10, 11). X indicates five informative recombinants from three independent backcrosses. The loci for *Lc* and *Lc*^l are shown below the top line, which represents chromosome 6. The *Lc* locus is flanked by two closest markers, *D6Rck353* and *D6Rck357*; *D6Rck354* and *D6Rck355* are non-recombinant with *Lc*. YACs and BACs are represented by lines proportional to the size of their insert, and relevant BACs are labelled (abbreviated names). The 5' and 3' ends of the 3024-bp coding sequence of the glutamate receptor δ2 subunit (*GluRδ2*) are represented by vertical bars. Three exons (A, B and C) were found within the *Lc* non-recombinant region. The detailed intron–exon structure of *GluRδ2* was not investigated further and so is represented by broken lines. Cen, centromere; Tel, telomere.

cells in response to NMDG substitution was $1,138 \pm 281$ pA ($n = 7$) compared to only 126 ± 1 pA in wild-type cells ($n = 6$, $P = 0.0088$). Furthermore, the average change in conductance produced by NMDG substitution was -12.3 ± 2.1 nS in *Lc/+* ($n = 7$) and -3.7 ± 1.2 nS in wild-type Purkinje cells ($n = 6$, $P = 0.0098$), suggesting that the positive change in holding current was caused by a decrease in inward current rather than an increase in outward current. These results show that the *Lc*-specific conductance is selective and is not the result of poor *Lc/+* membrane integrity or leakage at the pipette-membrane interface. Further, the large reduction in holding current magnitude and decrease in membrane conductance caused by reducing the external Na^+ concentration strongly suggests that Na^+ is a major current carrier of the *Lc*-specific, constitutive, inward current. Addition of $100 \mu\text{M}$ AP5, an NMDA receptor antagonist, and $10 \mu\text{M}$ CNQX, an AMPA/kainate receptor antagonist, to the external saline produced no significant change in either holding current ($-2,093 \pm 583$ pA before versus $-2,288 \pm 559$ pA after addition of drugs, $n = 3$, $P = 0.85$) or membrane conductance (36.1 ± 5.0 nS before versus 33.6 ± 6.3 nS after, $n = 3$, $P = 0.81$). These experiments were performed in the presence of tetrodotoxin, a potent voltage-gated Na^+ -channel blocker that should eliminate the release of glutamate evoked by any action potentials arising spontaneously within the slice preparation. Taken together, these results suggest that the

constitutively active current in *Lc/+* Purkinje cells does not depend on activation by ambient transmitter.

To determine whether this constitutive inward current in *Lc/+* Purkinje cells is a direct consequence of mutant *GluR δ 2^{Lc}*, we further examined the properties of the mutant channel in *Xenopus* oocytes. Consistent with previous reports^{7,8}, wild-type *GluR δ 2*-expressing oocytes were not significantly different from their uninjected counterparts in resting potential observed either in the absence or presence of NMDG (Fig. 4a). In contrast, injection of *GluR δ 2^{Lc}* cRNA, which was prepared by changing a single nucleotide from G to A at position 1,960 in the full-length cDNA sequence to recreate the *GluR δ 2^{Lc}* allele, produced a dramatic depolarization in the resting potential. This constant depolarization could be completely reversed by replacement of external Na^+ with NMDG (Fig. 4a). To investigate further the properties of the *GluR δ 2^{Lc}*-expressing oocytes, current-voltage relationships were obtained in the presence and absence of NMDG. As expected, there was no significant change in current in a typical oocyte expressing the wild-type *GluR δ 2* channel at any membrane potential measured. In contrast, substitution of NMDG for extracellular Na^+ in a representative *GluR δ 2^{Lc}*-injected oocyte resulted in a large change in the observed current at negative membrane potentials (Fig. 4b). As shown in Fig. 4c, quantitative analysis of the change in whole-cell conductance at -60 mV of 15 oocytes injected with the mutant

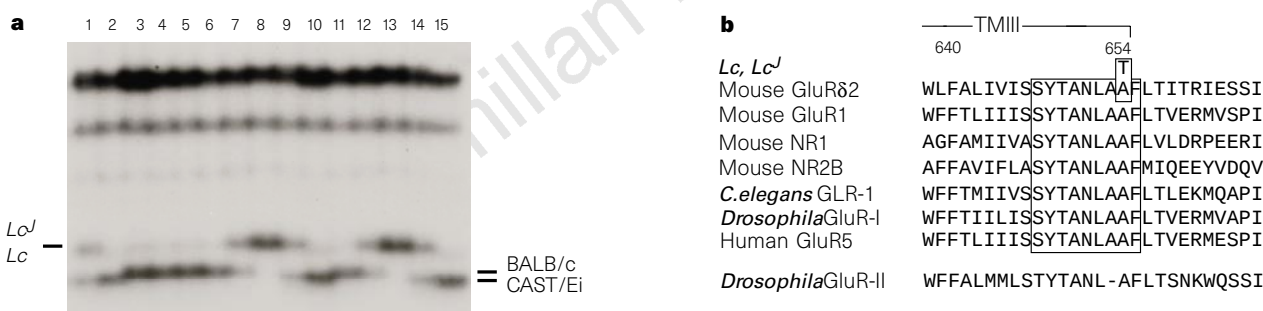


Figure 2 Mutation in the *GluR2* gene in *Lc* and *LcL*. **a**, Analysis of genomic DNA by SSCP. Samples were amplified with two exon primers flanking exon B. Samples in lanes 1–15 are designated as follows: 1, *Lc*/CAST/Ei; 2, CAST/Ei; 3, DBA/2J; 4, C3HeB/F₁; 5, C57BL/6J; 6, B6CBACa^{W-J}/A; 7, B6CBACa^{W-J}/A-*Lc*; 8, *Lc*/*Lc*; 9, *Lc*/CAST/Ei; 10, CAST/Ei; 11, BALB/cByJ; 12, *Lc*/BALB/cByJ; 13, *Lc*/*LcL*; 14, *LcL*/CAST/Ei; 15, CAST/Ei. In addition to two invariable bands at the top, a detectable polymorphism between CAST/Ei and other inbred strains, labelled only as BALB/c at the bottom, is detected. An abnormal band specific to *Lc* and *LcL* is labelled as *LcL*, *LcL* in the middle. **b**, Conservation of the alanine (A) residue in members of the ionotropic GluR family in murine and other species. The *Lc* and *LcL* mutation results in an Ala-to-Thi substitution at amino-acid position 654 (small box) of mouse *GluR2* (Genbank accession no. D13266) in a highly conserved domain (large box) of nine amino acids in TMIII. Five other representative members of the ionotropic glutamate receptor family include: mouse *GluR1* (X57497); mouse NR1 (D10028); mouse NR2B (D10651); *Caenorhabditis elegans* GLR-1 (U34661); *Drosophila* (M97192); human *GluR5* (L19058). An exception is *Drosophila* *GluR-II* (M73271).

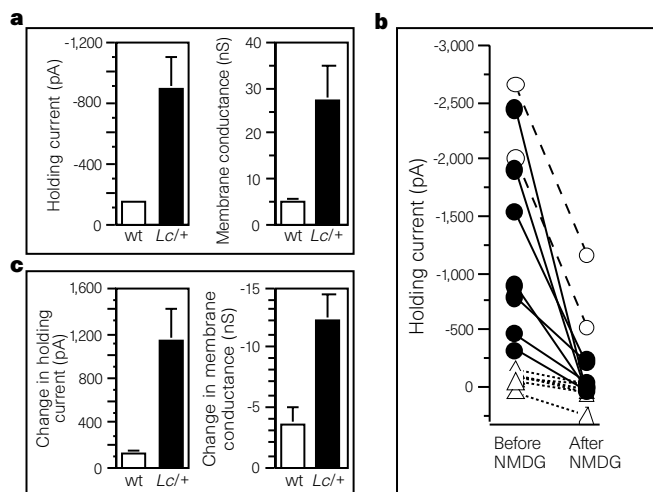


Figure 3 *Lc/+* Purkinje cells express a large, constitutively active and selective resting conductance. **a**, Average values of the holding current and membrane conductance in Purkinje cells from wild-type (WT) and *Lc/+* mice were determined on first entering whole-cell mode. **b**, Holding current before and after exchange of normal extracellular saline (containing 154 mM Na^+) with NMDG-substituted saline (containing 27 mM Na^+ and 138 mM NMDG) are plotted for individual *Lc/+* and wild-type recordings. Filled circles, 'healthy' *Lc/+* recordings ($n = 7$). Open circles, 'compromised' *Lc/+* recordings ($n = 2$). Open triangles, healthy wild-type recordings ($n = 6$) (See Methods). **c**, Average changes in holding current and membrane conductance induced by NMDG substitution. The large magnitude of the changes is specific to *Lc/+* Purkinje cells.

GluR $\delta 2^{Lc}$ cRNA in the presence and absence of NMDG reveals a substantial conductance change. In contrast, no significant change in conductance could be measured in five oocytes injected with the wild-type GluR $\delta 2$ cRNA or in four uninjected oocytes.

These changes in resting potential and whole-cell conductance were observed in the absence of any ligand, demonstrating that cells injected with mutant GluR $\delta 2^{Lc}$ expressed a large, constitutive conductance under physiological conditions. Because this conductance was significantly decreased by substitution of NMDG for extracellular Na⁺, the large inward currents observed in GluR $\delta 2^{Lc}$ -expressing oocytes at hyperpolarized membrane potentials were mostly caused by a constitutive inward Na⁺ current. Replacement of external Na⁺ with isomolar K⁺ resulted in a small increase in the amplitude of inward current at hyperpolarized membrane potentials (data not shown), indicating that the constitutive conductance in GluR $\delta 2^{Lc}$ -expressing oocytes is also permeable to K⁺. Application of ionotropic glutamate receptor agonists, 1 mM glutamate, 1 mM aspartate and 0.1 mM kainate in the presence of 10 μ M glycine, or the antagonists, 0.1 mM CNQX, 0.1 mM AP5 and 0.1 mM 7-chlorokynurenic acid, had no effect on the GluR $\delta 2^{Lc}$ current (data not shown). The constitutive current observed in both Purkinje cells and oocytes is therefore probably due to the increased conductance

of the GluR $\delta 2^{Lc}$ channel, although it remains possible that this is a secondary effect of the mutant channel.

The identification of the *Lc* mutation in GluR $\delta 2$ explains both the selectivity and the timing of *Lc* gene action in the cerebellum. Previous studies of GluR $\delta 2$ (refs 7, 8, 13–16) have shown that it is expressed selectively in cerebellar Purkinje cells and in some hindbrain nuclei, consistent with their selective vulnerability in *Lc/+* and *Lc/Lc* animals. Although GluR $\delta 2$ expression in Purkinje cells normally begins during embryonic development¹³, an increase in GluR $\delta 2$ expression and its redistribution to Purkinje cell/parallel fibre synapses seems to be concurrent with the onset of *Lc/+* phenotypic abnormalities in cerebellum¹³, slightly preceding the large-scale death of *Lc/+* Purkinje cells during the second and third postnatal weeks. Our results also suggest that apoptotic cell death in response to *Lc* mutation in GluR $\delta 2$ may be related to excitotoxic cell death in response to excess exposure to glutamate¹⁷. Given the central role of increased concentrations of intracellular calcium in initiating cell death in hypoxic or excitotoxic injury¹⁷, it is of interest to assess the contribution of intracellular Ca²⁺ levels in *Lc* Purkinje cell death. The identification of several other mouse neurological mutant genes as ion channels highlights the importance of membrane excitability during the development and degeneration of neurons in the mammalian central nervous system^{18–21}. Thus studies of downstream pathways mediating *Lc/+* Purkinje cell death will be directly relevant for our understanding of the mechanisms of mammalian neurodegeneration.

Methods

Mice and crosses. All mouse strains were either purchased from or provided by the Jackson Laboratory. The genetic map was based on three independent intersubspecific backcrosses^{11–12}. The five informative recombinants are: CL230 on the centromeric side, and CL103, CL369 and CL391 on the telomeric side from the first backcross; and CJ28 on the telomeric side from the second and third backcrosses. All progeny from the *Lc/CAST/Ei* and *L^d/CAST/Ei* intercrosses were genotyped with *D6Rck330* and *D6Rck361* markers, and occasionally with other markers such as *D6Rck357* and *D6Mit94* (refs 10, 11).

Identification of the GluR $\delta 2$ gene. Three overlapping BACs (207L3, 251E15 and 222L15) that span the *Lc* region were shotgun subcloned and sequenced. We identified a 300-bp exon from the mouse GluR $\delta 2$ (exon A in Fig. 1; 1,550–1,850 of the coding sequence). The exon-trapping technique was applied independently to two BACs (207L3 and 143E21; Life Technology, 18449-017) and a 147-bp exon (exon B in Fig. 1; 1851–1998) was identified. A mouse cDNA clone encoding GluR $\delta 2$ was isolated from an adult mouse brain cDNA library and mapped onto the YAC/BAC contig and genetic map (Fig. 1). A third exon (exon C in Fig. 1; 1999–2194) was identified by screening the subcloned libraries of BACs with the full-length cDNA probe. Exon A was confirmed to be non-recombinant with *Lc* by restriction fragment length polymorphism (RFLP) analysis using a *Bam*HI polymorphism (data not shown).

Mutation analyses. SSCP analyses were performed as described previously¹⁰. For RT-PCR SSCP, primer pairs were chosen to provide 2 $\times$ coverage from nucleotides 1200–2518 in the coding sequence of GluR $\delta 2$. The intron primer pair for exon A is: 5'-CACACTACTAATGTATTCTC-3' and 5'-CACTGCTGTGAAGATCATTAAGAG-3'. The intron primer pairs for exon B are: 5'-TAAAAGCATATTGATGTGTG-3' or 5'-GCACTGAATGTGTATGACTTCAG-3', and 5'-CAGCATTTGTCAGGTTTGGTGAC-3'. Exon primers for exon B were designed to amplify sequence at 1859–1998. The intron primer pair for exon C is: 5'-CTTAGAGGCAGTCCTCCTTAGC-3' and 5'-GAGAC-TATTGATGAAGAGCACAG-3'. Three different combinations of intron and exon primers were used for genomic SSCP analyses of exon B. Sequences were obtained by direct sequencing from PCR products in an ABI 377 automated sequencer following the standard protocol. For RT-PCR sequencing, sequences from nucleotides 1,687 to 2,022 were obtained from six different RNA templates of *Lc* and *L^d* heterozygotes, and sequences from 973 to 2,518 were obtained from postnatal day 12 (P12) cerebellar RNA of *Lc/CAST/Ei* mice. For genomic PCR sequencing, exon B was amplified with the two pairs of exon and intron primers listed above, and PCR products from all genomic samples listed in Fig. 2a were sequenced with two PCR primers and one internal exon

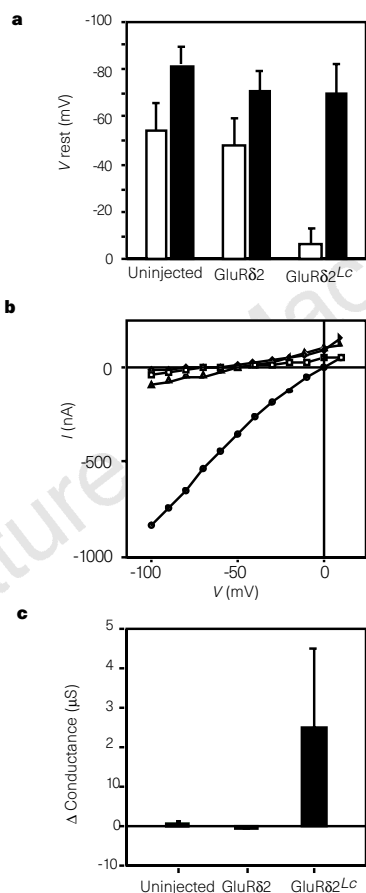


Figure 4 A large, constitutive conductance of GluR $\delta 2^{Lc}$ expressed in *Xenopus* oocytes. Error bars in **a** and **c** represent s.e.m. of two electrode voltage measurements from 4 uninjected oocytes, 5 oocytes injected with wild-type GluR $\delta 2$ cRNA, and 15 oocytes injected with mutant GluR $\delta 2^{Lc}$ cRNA. **a**, Average resting membrane potentials (V_{rest}) of uninjected oocytes and oocytes injected with GluR $\delta 2$ and GluR $\delta 2^{Lc}$ cRNAs in external Na⁺ bath (open bars) and in external NMDG bath (filled bars). **b**, Current-voltage relationships of two representative oocytes before and after NMDG substitution. Open squares (Na⁺) and diamonds (NMDG) are from the oocyte injected with GluR $\delta 2$ cRNA; filled circles (Na⁺) and triangles (NMDG) are from the oocyte injected with GluR $\delta 2^{Lc}$ cRNA. **c**, Average changes in whole-cell conductance at -60 mV membrane potential before and after NMDG substitution.

primer. A sequence change from T to G at nucleotide position 1,908 occurred in CAST/Ei relative to inbred strains, such as DBA/2J, C3HeB/FeJ, C57BL/6J, and BALB/cByJ (data not shown); however, this polymorphism does not change the coding of this alanine residue and could account for the non-*Lc*-specific difference seen in Fig. 2a. At nucleotide position 1,960, all sequences consistently show single peaks of A in all *Lc* and *Lc^d* homozygous samples, or double peaks of G and A in all *Lc* and *Lc^d* heterozygous samples.

Electrophysiology in cerebellar Purkinje cells. Slices (200 μ m thick) of cerebellar vermis were prepared from *Lc/+* and *Lc^d/+* mice (10 and 11 days old) and their wild-type littermates. Purkinje cells were whole-cell patch-clamped at a command potential of -70 mV using standard methods. External saline consisted of (in mM): 127 NaCl, 2.5 KCl, 2 CaCl₂, 1 MgCl₂, 1 NaH₂PO₄, 26.3 NaHCO₃, 20 glucose, 0.02 bicuculline, 0.005 tetrodotoxin, pH ~ 7.35 , 320 mmol kg⁻¹. For NMDG-substituted external saline, all NaCl in the above formulation was replaced with 138 mM NMDG and the pH was adjusted with HCl, reducing external Na⁺ concentration to 27.3 mM (thereby changing E_{Na} from about $+67.4$ mV to $+23.2$ mV). Internal solution of (in mM): 15 KCl, 135 potassium gluconate, 1 MgCl₂, 10 HEPES, 4 Na₂ATP, 1 Na₃GTP, 10 sucrose, pH ~ 7.25 , 300 mmol kg⁻¹. Series and input resistances were derived from membrane current responses to 125-ms voltage steps to -75 mV.

Fewer Purkinje cells were visible in *Lc/+* than in wild-type cerebellar slices, and those that were present appeared shrivelled and granular. In mutant slices, those Purkinje cells that appeared most healthy were chosen for electrophysiological analysis. The two alleles, *Lc/+* and *Lc^d/+*, were indistinguishable in electrophysiological recordings and results were combined. *Lc/+* recordings segregated into two groups based on their final holding currents following NMDG substitution. In most *Lc/+* Purkinje cells (Fig. 3b, filled circles, $n = 7$), post-NMDG holding currents were very small. In a few *Lc/+* cells however, the final holding currents in the presence of NMDG were very large despite the decrease observed in response to this substitution (Fig. 3b, open circles, $n = 2$). This large residual holding current was probably a sign of compromised recording integrity, evidence of which had initially been masked by the *Lc/+* constitutive current. Thus data from these cells were excluded from the mean calculations of both basic *Lc/+* membrane properties (holding current and conductance; Fig. 3a) and changes in these values produced by NMDG substitution (Fig. 3c) to avoid artefactually increasing the *Lc/+* effect. The values we report here, therefore, were derived only from those *Lc/+* cells for which the post-NMDG holding currents were more positive than -230 pA.

Electrophysiology in *Xenopus* oocytes. Two oligonucleotides were designed with homology to the 5' and 3' ends of the mouse *GluR δ 2* cDNA coding sequence. The *Lc* mutation was generated using the bridge PCR mutagenesis method²². The *Lc* mutation in mutant clones was verified by sequencing. *In vitro* translation was used to confirm that both wild-type and mutant constructs yielded the expected products of $M_r \sim 110$ K in an SDS-PAGE gel. Concentrations of cRNAs were measured by gel electrophoresis and spectrophotometer. Approximately 65–85 ng per 50 nl of the cRNAs were injected into stage V or VI oocytes from *Xenopus laevis* (Nasco and *Xenopus* Express). Electrophysiological recordings were performed 1–3 days postinjection using the two-microelectrode voltage-clamp configuration at room temperature²³. The recording-bath chamber was ~ 150 μ l in volume and the flow rate 2–3 ml min⁻¹. Most recordings were made in a Na/Ca solution containing (in mM): 130 NaCl, 2 CaCl₂ and 10 HEPES, pH 7.3. For NMDG substitution experiments, an equimolar substitution of NMDG for NaCl was made (pH 7.3, adjusted with HCl). Fresh stock solutions were made in the Na/Ca recording solution and kept on ice: L-glutamate (100 mM, Sigma), L-aspartate (100 mM, Sigma), glycine (100 mM, Sigma), kainic acid (1 mM, Research Biochemicals), CNQX (1 mM, Research Biochemicals), AP5 (1 mM, Research Biochemicals), 7-chlorokynurenic acid (10 mM, Research Biochemicals). For resting-potential measurements, close readings between two microelectrodes (less than 5 mV difference) were indicating of good seals of the microelectrodes to the oocyte membrane. When NMDG bath was perfused after Na⁺ bath, the readings of two microelectrodes moved very closely to more hyperpolarized or negative directions until they reached a relatively stable state, between -50 and -90 mV. Some oocytes did not reach such a hyperpolarized state with NMDG, either before or after changes of external baths for measurements, usually indicating compromised membrane integrity, so these oocytes were not further studied. For current-voltage relationships, oocytes were held at their resting potentials

and stimulated with voltage steps of 50 ms and 10 mV from -140 to $+100$ mV, each pulse being separated by 2 s. Currents were measured after 25-ms during each pulse. Conductance was derived from the difference between the holding currents at -50 and at -60 mV.

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A second endogenous cannabinoid that modulates long-term potentiation

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Cannabinoid receptors are molecular targets for marijuana and hashish, the widespread drugs of abuse. These receptors are expressed in areas of the central nervous system that contribute in important ways to the control of memory, cognition, movement and pain perception¹. Indeed, such functions can be strongly influenced by cannabinoid drugs, with consequences that include

euphoria, analgesia, sedation and memory impairment². Although the pharmacology of cannabinoid drugs is now beginning to be understood, we still lack essential information on the endogenous signalling system(s) by which cannabinoid receptors are normally engaged. An endogenous ligand for cannabinoid receptors, anandamide, has been described³. Here we report that *sn*-2 arachidonylglycerol (2-AG), a cannabinoid ligand isolated from intestinal tissue⁴, is present in brain in amounts 170 times greater than anandamide. 2-AG is produced in hippocampal slices by stimulation of the Schaffer collaterals, an excitatory fibre tract that projects from CA3 to CA1 neurons. Formation of 2-AG is calcium dependent and is mediated by the enzymes phospholipase C and diacylglycerol lipase. 2-AG activates neuronal cannabinoid receptors as a full agonist, and prevents the induction of long-term potentiation at CA3–CA1 synapses. Our results indicate that 2-AG is a second endogenous cannabinoid ligand in the central nervous system.

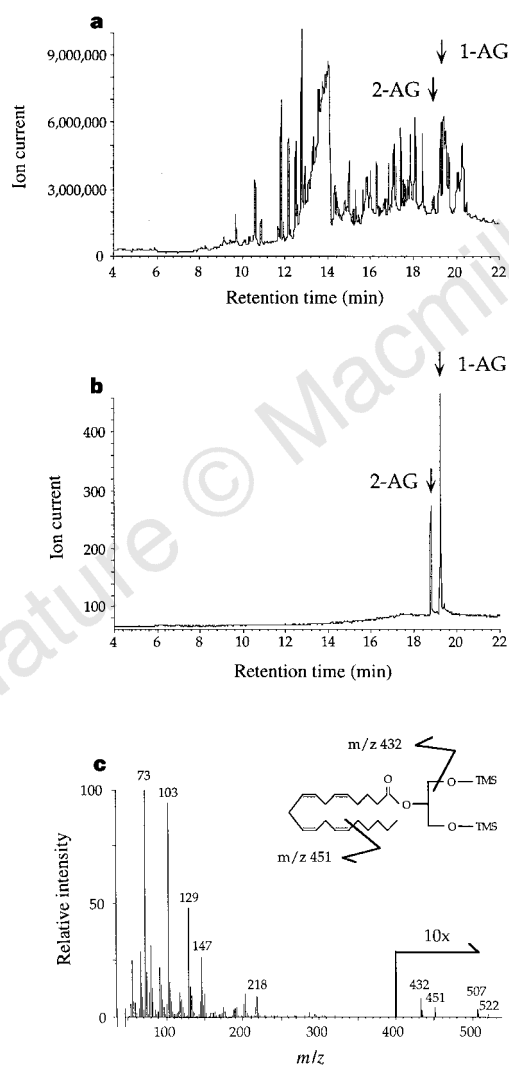


Figure 1 Identification of 2-arachidonylglycerol (2-AG) in rat brain by GC/MS. Monoacylglycerols were purified chromatographically and analysed by GC/MS as bis trimethylsilylether (TMS) derivatives. **a**, Total ion current chromatogram of HPLC-purified material from one brain. Arrows indicate the retention times of synthetic 2-AG and 1(3)-AG. **b**, Selected-ion monitoring (SIM) analysis. **c**, Electron-impact mass spectrum of 2-AG purified from brain, which was identical to that of synthetic 2-AG (data not shown). Results are from one experiment, representative of four.

Anandamide fulfils several criteria of an endogenous cannabinoid substance: (1) it is present in brain and activates cannabinoid receptors³; (2) it is released from depolarized neurons^{5,6}; and (3) it undergoes rapid biological inactivation^{5,7–10}. Nevertheless, the exceedingly low levels of anandamide in brain^{11–14} and the discrepancies observed between its pharmacological properties and those of other cannabinoid drugs¹⁵ indicate that additional cannabinoids may exist. In support of this possibility, cannabinoid receptor ligands that are chemically distinct from anandamide have been found in tissues^{4,16}. One such ligand was isolated from dog intestine and characterized as *sn*-2 arachidonylglycerol (2-AG)⁴. 2-AG binds to cannabinoid receptors and exerts several effects typical of cannabinoid drugs^{4,17}. Yet the possible role of 2-AG as an endogenous cannabinoid remains unknown; even its occurrence in brain is at present controversial^{4,17}.

To determine whether 2-AG is present in brain, we analysed lipid extracts of rapidly frozen rat brains using gas chromatography/mass spectrometry (GC/MS). A component was eluted from the GC at the retention time of 2-AG (analysed as bis trimethylsilylether derivative; Fig. 1a, b, $n = 4$). We identified this component from its electron-impact mass spectrum (Fig. 1c). Diagnostic fragments included m/z 522 ($[M]^+$, molecular ion), 507 ($[M-15]^+$, loss of methyl radical), 451 ($[M-71]^+$, loss of pentyl radical), and 432 ($[M-90]^+$, loss of trimethylsilenol). Such fragmentation is typical of *sn*-2 acylglycerols, and identical to that of synthetic 2-AG (data not shown). The identification of 2-AG was further supported by GC/

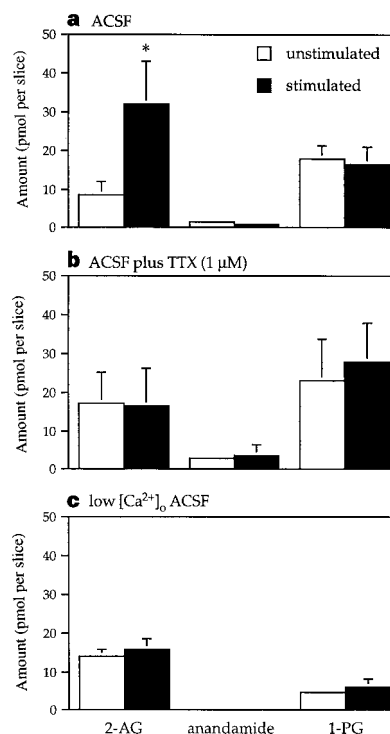


Figure 2 Neural activity stimulates 2-AG production in hippocampal slices. Effects of high-frequency stimulation (HFS) on the production of 2-AG, anandamide and 1(3)-palmitoylglycerol (1-PG) in: **a**, artificial cerebrospinal fluid (ACSF); **b**, ACSF containing tetrodotoxin (TTX; 1 μ M); **c**, ACSF with zero Ca^{2+} and 6 mM Mg^{2+} . Unstimulated slices (white bars) and stimulated slices (black bars) were immersed in cold methanol after the experiment and lipids were extracted with chloroform. Acylglycerols and anandamide were purified by HPLC and analysed by GC/MS in the SIM mode as bis TMS derivatives. Average weight of the slices was 30.9 ± 1.3 mg, $n = 20$. Results represent the mean $\pm$ s.e.m. of 3–7 experiments, each done on 4 slices. *, $P < 0.05$ significantly different from unstimulated controls (Wilcoxon non-parametric test).

MS analysis of the bis trifluoroacetate derivative, which yielded the following diagnostic fragments: $[M-43]^+$, loss of propyl radical; $[M-71]^+$, loss of pentyl radical; $[M-98]^+$, loss of trifluoroacetate. By comparison with an internal standard, we estimate that 2.4 ± 1.3 nmol of 2-AG were recovered per g of brain tissue (mean $\pm$ s.e.m.; $n = 3$).

Figure 1b also shows another GC component, which we identified as 1(3)-AG from its retention time and mass spectrum (data not shown). Although we recovered a substantial amount of 1(3)-AG (1.6 ± 0.6 nmol g⁻¹, 40% of total AG, $n = 3$), this could be entirely accounted for by non-enzymatic isomerization of 2-AG. Indeed, when we subjected synthetic 2-AG to sample preparation, we found that $43.3 \pm 5.6\%$ was isomerized to 1(3)-AG ($n = 6$). Considering this reaction, we estimate that the content of 2-AG in brain is 4.0 ± 1.8 nmol g⁻¹. We made a similar correction in all subsequent measurements.

Because we used brains frozen within 10 s of decapitation, the levels of 2-AG obtained in our experiments are unlikely to reflect postischaemic lipid breakdown¹⁸. This conclusion was confirmed by measuring anandamide, which is sensitive to ischaemic challenge¹². We measured 23 ± 3 pmol of anandamide per g of brain, a value that tallies well with those previously reported^{11–14}. These analyses demonstrate that 2-AG is a prominent constituent of brain tissue, about 170 times more abundant than anandamide.

We determined whether neural activity affects 2-AG production by electrically stimulating superfused slices of hippocampus. High-frequency stimulations (HFS) of the Schaffer collaterals, an excitatory CA1 afferent, elicited robust responses in CA1. Without stimulation, we measured 8.6 ± 3.5 pmol of 2-AG per slice ($n = 6$). HFS produced a fourfold increase in 2-AG levels, to

32.0 ± 11.2 pmol per slice (Fig. 2a). This effect was prevented by the Na⁺ channel blocker tetrodotoxin (1 μ M) or by removal of Ca²⁺ ions, indicating that 2-AG formation required both neuronal depolarization and external Ca²⁺ (Fig. 2b, c). Moreover, HFS was specific in stimulating 2-AG production, as the levels of 1(3)-palmitylglycerol and 1(3)-stearylglycerol were not significantly changed (Fig. 2a–c and data not shown). In cultured neurons, anandamide formation is increased by membrane-depolarizing agents^{5,6}. However, HFS had no significant effect on anandamide levels in hippocampal slices. Anandamide was 1.5 ± 0.7 pmol per slice before, and 0.8 ± 0.4 pmol per slice after stimulation ($n = 7$; Fig. 2a).

HFS increased 2-AG production only when Ca²⁺ was present in the superfusing medium. This suggests that Ca²⁺ stimulates enzyme activities involved in 2-AG production or that Ca²⁺ is required for the release of neurotransmitters that elicit 2-AG production. To address these possibilities, we studied 2-AG formation in serum-free cultures of rat brain neurons, which lack Ca²⁺-dependent neurotransmitter release¹⁹. We labelled the neurons with [¹⁴C]arachidonic acid (AA) and analysed the lipids by thin-layer chromatography (TLC). Figure 3a shows that [¹⁴C]2-AG was accumulated in response to the Ca²⁺-ionophore ionomycin (2 μ M), and reduced by chelating external Ca²⁺ with EGTA (1 mM). Thus, Ca²⁺ may act directly to increase 2-AG formation in neurons. We then determined whether astroglia may also contribute to 2-AG formation. Under basal conditions, cultures of brain astrocytes labelled with [¹⁴C]AA contained only small amounts of [¹⁴C]2-AG. [¹⁴C]2-AG was reduced below detection by EGTA, but was not affected by ionomycin even though the Ca²⁺ ionophore significantly increased the levels of non-esterified [¹⁴C]AA (Fig. 3b). Thus, although

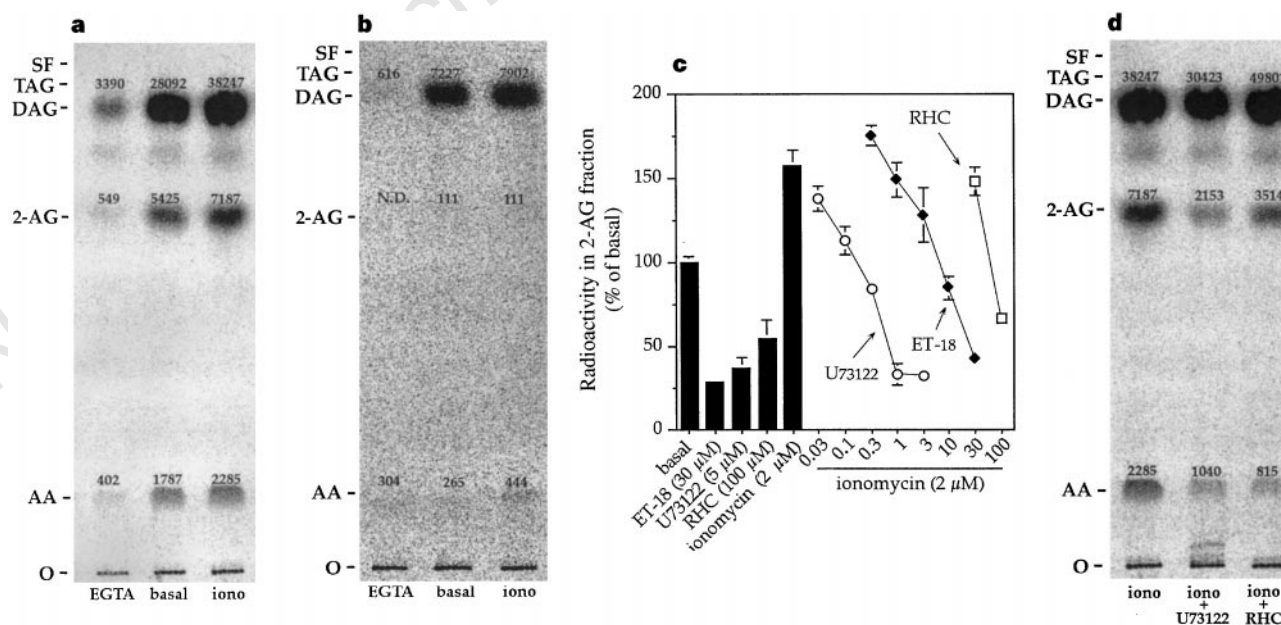


Figure 3 Ca²⁺-dependence, cellular localization and role of PLC and DAG lipase activities in 2-AG formation. **a**, Ca²⁺-dependent production of [¹⁴C]2-AG in cultured neurons labelled overnight with [¹⁴C]arachidonic acid (AA). Representative TLC analysis of samples from 10-min incubations with 1 mM EGTA (EGTA), no additions (basal), or 2 μ M ionomycin (iono). The bars indicate the chromatographic mobilities of AA, 2-AG, diacylglycerol (DAG) and triacylglycerol (TAG); the numbers represent average radioactivity in each component (d.p.m. mg⁻¹ protein, s.e.m. $\leq$ 10%, $n = 6$); O, origin; SF, solvent front. The effect of ionomycin on [¹⁴C]2-AG accumulation was time-dependent: 5 min, $104 \pm 3\%$ of control; 10 min, $162 \pm 18\%$; 20 min, $188 \pm 10\%$ ($n = 4$). Under all conditions, specific radioactivity in [¹⁴C]2-AG was ~ 50 pCi pmol⁻¹. **b**, Effects of EGTA (1 mM) and ionomycin (2 μ M) on [¹⁴C]2-AG production in cortical astrocytes. Representative TLC analysis and average radioactivities from 4 experiments (s.e.m. $\leq$ 20%). [¹⁴C]AA under basal conditions was increased significantly by the addition of ionomycin; $P < 0.05\%$ (unpaired Student's *t*-test); nd, not detectable. **c**, Effects of PLC inhibitors (U73122, ET-18) and DAG lipase inhibitor (RHC) 80267 on [¹⁴C]2-AG production in cortical neurons. The drugs were applied alone (bars) or in the presence of 2 μ M ionomycin (circles, U73122; diamonds, ET-18; squares, RHC 80267). Results are expressed as percentage of basal [¹⁴C]2-AG production (which was $5,425 \pm 149$ d.p.m. mg⁻¹ protein) and represent the mean $\pm$ s.e.m. of 6 experiments. **d**, Effects of PLC and DAG lipase inhibitors on ionomycin-induced formation of [¹⁴C]DAG, [¹⁴C]2-AG and [¹⁴C]AA in neurons. Representative TLC analysis and average radioactivities from 6 experiments (s.e.m. $\leq$ 10%). Concentrations of U73122 and RHC 80267 were 5 μ M and 100 μ M, respectively.

astrocytes may produce 2-AG, the Ca^{2+} -dependent formation of this lipid may be less prominent in astrocytes than in neurons. 2-AG may be produced through three main biochemical routes. In intestine, 2-AG accumulates during the digestion of dietary triacylglycerols²⁰. In other tissues, 2-AG formation may result from the cleavage of phospholipids in membranes (initiated by phospholipase C; PLC)²¹ or from the breakdown of triacylglycerols (initiated by neutral lipase)²⁰. The PLC inhibitor U73122 is potent in preventing the accumulation of [^{14}C]2-AG in neurons (Fig. 3c, d), with a median inhibitory concentration (IC_{50}) of 0.2 μM . To confirm these results and test which PLC isoenzyme(s) may be implicated, we used two additional PLC inhibitors: ET-18, which blocks phosphoinositide-specific PLC, and D-609, which blocks phosphatidylcholine-specific PLC. ET-18 prevented [^{14}C]2-AG formation with an IC_{50} of 5 μM (Fig. 3c), whereas D-609 was ineffective even when used at 100 μM (ionomycin, $170 \pm 12\%$ of basal; ionomycin plus D-609, $242 \pm 37\%$ of basal, $n = 6$).

The primary lipid product of PLC activity is diacylglycerol (DAG), which is then hydrolysed to *sn*-2 acylglycerol by DAG lipase activity²². If 2-AG production in neurons is initiated by PLC, 2-AG should accumulate in parallel with DAG. Accordingly, [^{14}C]DAG and [^{14}C]2-AG levels increased by a similar extent when neurons were incubated in a medium containing Ca^{2+} (basal) or Ca^{2+} plus ionomycin (Fig. 3a). We obtained further evidence that DAG serves as a precursor for 2-AG using the DAG lipase inhibitor RHC 80267. At 100 μM , this drug reduced both [^{14}C]2-AG formation (Fig. 3c, d) and [^{14}C]DAG hydrolysis (Fig. 3d), whereas it had no effect on [^3H] inositol phosphate accumulation (data not

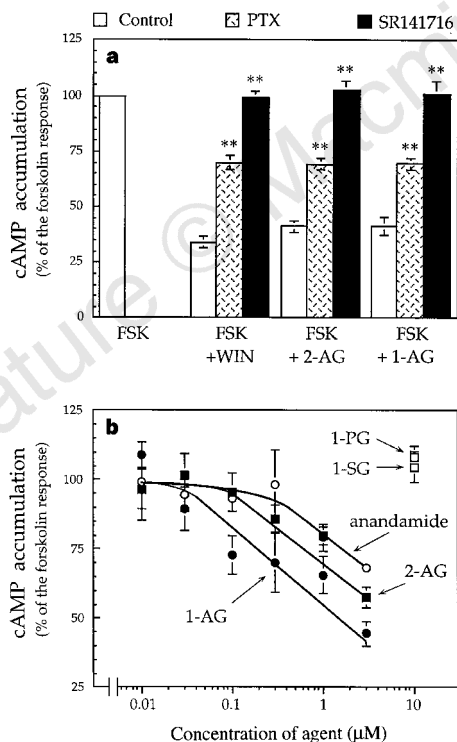


Figure 4 Activation of neuronal CB1 receptors by 2-AG. **a**, Inhibitory effects of WIN 55212-2 (WIN, 3 μM), 2-AG (10 μM) or 1(3)-AG (10 μM) on forskolin-stimulated cAMP accumulation in rat cortical neurons (white bars). These effects were prevented by the CB1 receptor antagonist SR-141716 (1 μM) (black bars) or by pertussis toxin (PTX; 1 $\mu\text{g ml}^{-1}$) for 18–20 h (stippled bars). **, $P < 0.01$ (ANOVA followed by Dunnett's test). **b**, Concentration-dependent inhibition of cAMP accumulation by 2-AG, 1(3)-AG and anandamide, and lack of effect of 1(3)-palmitoylglycerol (1-PG) and 1(3)-stearylglycerol (1-SG). cAMP levels were measured in neurons after 10-min incubations in the presence of forskolin (3 μM) and 3-isobutyl-1-methylxanthine (1 mM), using a commercial radioimmunoassay kit (Amersham, Arlington, IL).

shown). These experiments suggest that 2-AG formation is mediated by PLC and DAG lipase activities. We cannot exclude, however, that other lipases such as PLA₁ or triacylglycerol lipase may also be involved.

We investigated next the functional consequences of 2-AG binding to CB1 cannabinoid receptors. Cortical neurons in culture express CB1 receptors that are negatively coupled to adenylyl cyclase activity through a G_i/G_o protein²³. Accordingly, the CB1 receptor agonist WIN-55212-2 (3 μM) inhibited forskolin-induced cAMP accumulation, an effect that was reverted by the CB1 antagonist SR-141716 (1 μM) or by pertussis toxin (Fig. 4a). 2-AG (10 μM) elicited a quantitatively similar inhibition which was also sensitive to SR-141716 and pertussis toxin (Fig. 4a). This effect occurred with

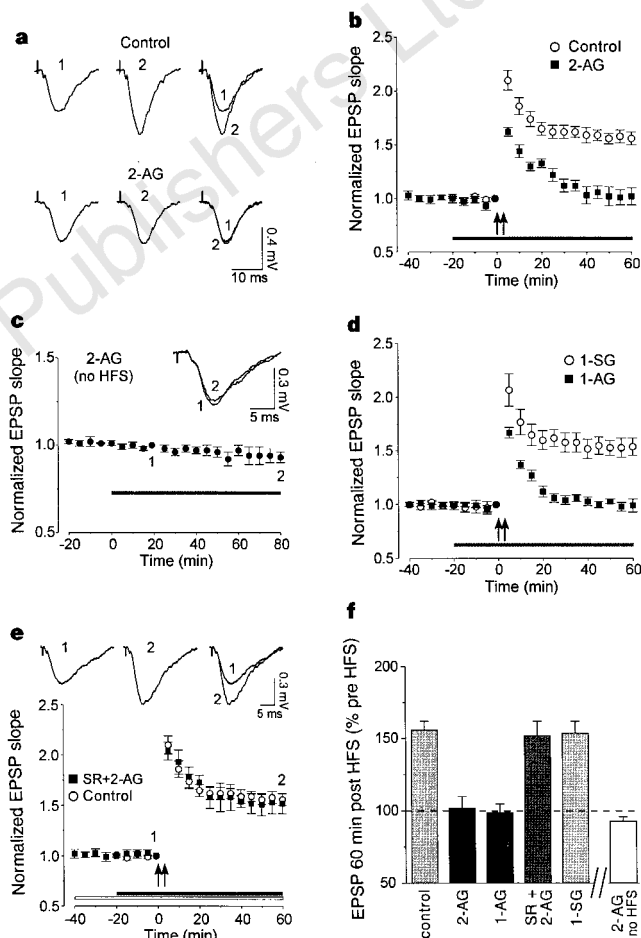


Figure 5 2-AG prevents the induction of LTP. **a**, Sample traces showing CA1 field excitatory postsynaptic potentials (EPSPs) (1) before and (2) 60 min after HFS of the Schaffer collaterals. The upper traces show control potentiation of EPSP slope and amplitude 60 min after HFS. The lower traces show inhibition of this potentiation by 2-AG (20 μM). **b**, Time course of field EPSP slope (normalized to pre-tetanus). 2-AG (20 μM , filled squares; $n = 6$) inhibited LTP (empty circles; $n = 7$). Drug application is denoted by the solid bars. HFS was delivered at the time shown by the double arrow. Results in this and all graphs represent mean $\pm$ s.e.m. **c**, 2-AG (20 μM ; $n = 4$) had no significant effect on EPSP in the absence of HFS. The sample traces depict EPSP obtained at times indicated by the numbers. **d**, 1(3)-Stearylglycerol (20 μM , empty circles; $n = 3$) did not affect LTP, whereas 1(3)-AG (20 μM , filled squares; $n = 5$) completely blocked LTP. **e**, LTP inhibition by 2-AG was prevented by prior application (white bar) of SR-141716 (SR, 2 μM , filled squares, $n = 3$) and LTP was similar to control (empty circles). The recordings depict EPSPs obtained in a slice treated with SR-141716 and 2-AG. **f**, Effects of pharmacological agents on EPSP potentiation 60 min after HFS. Values are expressed as percentage of the EPSP before HFS or an equivalent time for the white bar, which represents the EPSP slope in non-tetanzed slices after 80-min exposure to 2-AG (20 μM).

a potency ($IC_{50} = 0.8 \mu\text{M}$, Fig. 3b) consistent with the affinity of 2-AG for CB1 receptors ($K_i = 0.7\text{--}2.4 \mu\text{M}$)^{4,17} and comparable to the potency of anandamide ($IC_{50} = 1.2 \mu\text{M}$, Fig. 4b). 1(3)-AG also decreased cAMP accumulation, whereas 1(3)-palmitylglycerol and 1(3)-stearylglycerol had no effect (Fig. 4b). These results indicate that 2-AG activates neuronal CB1 receptors as a full agonist and with a potency similar to that of anandamide.

CB1 agonists inhibit hippocampal long-term potentiation (LTP)^{24,25}, a model for learning and memory, and impair short-term memory tasks that are associated with the firing of hippocampal cells^{26,27}. We therefore examined whether 2-AG affects LTP expression. HFS of the Schaffer collaterals caused a sustained increase in the slope of the field excitatory postsynaptic potentials (EPSP) recorded in CA1 stratum radiatum: the EPSP slope measured 60 min after HFS was $156 \pm 6\%$ of basal values measured before HFS (Fig. 5a, b). LTP was completely suppressed when 2-AG ($20 \mu\text{M}$) was administered before HFS ($102 \pm 8\%$ of basal; Fig. 5a, b). By contrast, 2-AG had little or no effect on EPSP when administered to non-tetanized slices (Fig. 5c, f). Moreover, 2-AG had no effect on established LTP ($160 \pm 7\%$ of basal, $n = 4$). The blockade of LTP by 2-AG probably resulted from the activation of CB1 receptors, because: (1) the active isomer 1(3)-AG, but not 1(3)-stearylglycerol, prevented LTP induction (Fig. 5d, f); and (2) 2-AG had no effect on LTP when applied together with the CB1 antagonist SR-141716 ($2 \mu\text{M}$) ($152 \pm 10\%$ of basal; Fig. 5e, f). These results suggest that 2-AG acting at CB1 receptors inhibits LTP induction without affecting basal synaptic transmission.

In conclusion, we have shown that neural activity and Ca^{2+} entry may evoke the formation of 2-AG which may, in turn, activate CB1 receptors in neurons and modulate the induction of LTP at CA3/CA1 synapses. Our experiments suggest therefore that 2-AG, like anandamide, may serve an endogenous cannabinoid role. However, 2-AG appears to differ from anandamide in at least two ways. First, the formation of 2-AG may be initiated by PLC activity, whereas that of anandamide may be catalysed by PLD activity^{5,11,28}. Second, 2-AG and anandamide may be produced under distinct physiological conditions or in distinct brain regions. Thus, despite their common ability to activate cannabinoid receptors, the physiopathological implications of these signalling molecules may be different. Such demarcation should be instrumental in identifying pharmacological agents that selectively interfere with discrete components of the endogenous cannabinoid system. □

Methods

Lipid analyses. The methods used for rapid brain freezing, methanol/chloroform extraction of the lipids, open-bed chromatography and normal phase high-performance liquid chromatography (HPLC) have been described previously¹⁴. TLC analyses were carried out on silica gel G plates, eluted with a solvent system consisting of the organic phase of ethyl acetate/methanol/water/ammonia (20:1:10:1, v/v). Synthetic standards were visualized by phosphomolybdic acid staining, and radioactive lipids were localized by autoradiography using a Phosphor-Imager 445 SI apparatus (Molecular Dynamics, Sunnyvale, CA). Radioactivity in TLC fractions was determined by liquid scintillation counting. For GC/MS analyses of acylglycerols as TMS derivatives¹⁴, we added 1(3)-eicosanoylglycerol (1.2 mol, Nu-Check Prep, Elysian, MN) to tissue or cell culture homogenates, as internal standard. The TMS derivative of 1(3)-eicosanoylglycerol was eluted from the GC at a retention time of 20 min, and its electron-impact mass spectrum displayed a series of informative ions which included m/z 515 [$\text{M}-15$]⁺ (loss of a methyl radical) and m/z 427 [$\text{M}-103$]⁺ (loss of [TMSOCH_2]⁺). The ion at m/z 427 was used in quantitative SIM analyses to calculate recoveries. After correction for recoveries, brain 2-AG was quantified by comparison with calibration curves constructed using synthetic 2-AG. The GC/MS isotope dilution method used to quantify anandamide as TMS derivative will be described in detail elsewhere (A. Giuffrida, R. Kuczenski and D.P., manuscript in preparation).

Cell cultures, radioactive labelling and incubations. Primary cultures of cortical neurons (5–7 days *in vitro*) or astrocytes (6–8 weeks *in vitro*) were

prepared as described previously^{6,29}. Cells maintained in 6-mm culture dishes were incubated for 18–20 h with [¹⁴C] AA (Amersham, 54 mCi mmol⁻¹; 0.05 $\mu\text{Ci ml}^{-1}$ with 0.01% BSA). The cells were rinsed twice for 10 min with a HEPES-bicarbonate buffer composed of (in mM): NaCl 120; KCl 5.0; CaCl_2 2.0; MgSO_4 1.0; NaH_2PO_4 1.0; glucose 10; NaHCO_3 5.0 and HEPES 20 (pH 7.4, 37°C). The cells were then incubated for 10 min in the same buffer containing drugs at the indicated concentrations. For lipid analyses, incubations were stopped by adding 2 ml of ice-cold methanol followed by chloroform extraction.

Hippocampal slice stimulations and LTP experiments. Slices of rat hippocampus were prepared as described previously³⁰. Briefly, transverse slices (350–400 μm thick) were cut and superfused ($2\text{--}3 \text{ ml min}^{-1}$) with warm, gassed (31°C, 95% O_2 , 5% CO_2) artificial cerebrospinal fluid (ACSF) of the following composition (mM): NaCl 130; KCl 3.5; NaH_2PO_4 1.25; MgSO_4 1.5; CaCl_2 2.0; NaHCO_3 24; glucose 10. We stimulated the Schaffer collaterals using a concentric bipolar tungsten electrode (0.1 ms pulses). To measure stimulation-dependent 2-AG production, the HFS consisted of 2 trains (100 Hz) of 1 s each applied 20 s apart at an intensity that evoked maximal field EPSPs (as determined in separate experiments); 4 hippocampal slices were placed in the chamber and consecutively stimulated. At the end of the stimulation period (2 min), we immersed the slices in 2 ml of ice-cold methanol containing 1(3)-eicosanoylglycerol (1.2 nmol) and [³H]₄ anandamide (1.2 nmol as internal standards). The slices were homogenized and subjected to lipid analysis (see above). For LTP experiments, we recorded EPSPs with an extracellular 3 M NaCl-filled glass electrode (resistance 1–3 M Ω) placed in CA1 stratum radiatum, while stimulating the Schaffer collaterals at an intensity sufficient to evoke a 40% maximal response. The same intensity was kept for the HFS, which consisted of 2 trains (100 Hz) of 1 s each, applied 20 s apart. Drugs were added to the ACSF from stock solutions in dimethyl sulfoxide. The final concentration of dimethyl sulfoxide was 0.1%. Preliminary GC/MS analyses showed that 30% of exogenously added 2-AG (Deva Biotech, Harbore, PA) was adsorbed to the superfusion apparatus. Therefore, to obtain a final concentration of 20 μM , we added 30 $\mu\text{mol l}^{-1}$ 2-AG to the ACSF.

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Placental abnormalities in mouse embryos lacking the orphan nuclear receptor ERR- β

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Classical endocrine studies have shown that steroid hormones are required for the maintenance of pregnancy and placental viability. The oestrogen-receptor-related receptor β (ERR- β) is an orphan member of the superfamily of nuclear hormone receptors¹. Although ERR- β is homologous to the oestrogen receptor and binds the oestrogen response element², it is not activated by oestrogens¹. Expression of ERR- β during embryogenesis defines a subset of extra-embryonic ectoderm that subsequently forms the dome of the chorion, suggesting that ERR- β may be involved in early placental development. Homozygous mutant embryos generated by targeted disruption of the *Estrrb* gene have severely impaired placental formation, and die at 10.5 days post-coitum. The mutants display abnormal chorion development associated with an overabundance of trophoblast giant cells and a severe deficiency of diploid trophoblast. The phenotype can be rescued by aggregation of *Estrrb* mutant embryos with tetraploid wild-type cells, which contribute exclusively to extra-embryonic tissues. Our results indicate that ERR- β has an important role in early placental development, and suggest that an inductive signal originating from or modified by the chorion is required for normal trophoblast proliferation and differentiation.

During early embryonic development in the mouse, expression of the gene encoding ERR- β is restricted to extra-embryonic tissues (Fig. 1). ERR- β transcripts are first detected in a subset of cells in

extra-embryonic ectoderm at 5.5 days post-coitum (d.p.c.) and become more prominently expressed at 6.0 d.p.c. (Fig. 1a–d). At 6.5 d.p.c., ERR- β is expressed by ectodermally derived regions of the amniotic fold (Fig. 1e, f); these cells then form the chorion, where ERR- β expression is present at 7.5 d.p.c. (Fig. 1g, h). ERR- β expression diminishes as the chorion fuses with the ectoplacental cone, and by 8.5 d.p.c. ERR- β transcripts are present only at the free margin of the chorion (Fig. 1i, j). ERR- β transcripts are not detected in extra-embryonic tissue at subsequent stages of development. This highly specific expression pattern suggests that ERR- β may be important for the formation or function of the chorion.

To assess the role of ERR- β in placental development, we have created homozygous null *Estrrb* mutants by homologous recombination. Targeted disruption of the *Estrrb* gene was achieved in R1 embryonic stem (ES) cells by replacement of the second *Estrrb* exon with a neomycin phosphotransferase cassette (Fig. 2a). Two independent clones containing the predicted mutant allele produced male chimaeric mice that transmitted the mutation through the germ line (Fig. 2b). Mice heterozygous for the mutation were viable and were bred to generate homozygous mutants. No homozygotes were found in 62 live-born progeny of heterozygous intercrosses, indicating that the *Estrrb* mutation caused embryonic lethality. Embryos were genotyped at various stages of gestation to determine when fetal death occurred; viable homozygous mutants were not detected later than 10.5 d.p.c. (Fig. 2c and Table 1). Embryos examined at 9.5 d.p.c. demonstrated growth failure as well as absence of chorioallantoic fusion (Fig. 3a). At age 10.5 d.p.c., *Estrrb*^{−/−} embryos had no detectable heartbeat and had started to undergo resorption (Fig. 3b). These results indicate that the *Estrrb* mutation was lethal at 9.5–10.5 d.p.c., and that *Estrrb* ablation was associated with abnormal placental development.

Histological examination showed that embryonic and extra-embryonic tissues in *Estrrb* homozygous mutants were normal at 6.5 d.p.c. (Fig. 3c, d). In particular, development of the ectoplacental cone and the extra-embryonic ectoderm appeared normal in the mutant embryos. At 7.5 d.p.c., the chorion of the *Estrrb* homozygous mutants was either absent or hypoplastic and the embryos displayed varying amounts of growth retardation (Fig. 3d, f). In all cases, the extra-embryonic tissues appeared viable. At 8.5 d.p.c., abnormalities were seen in the development of early placenta and the ectoplacental cone, as the *Estrrb* mutants showed a marked increase in the number of secondary giant cells accompanied by a near absence of diploid trophoblast cells. Further, chorioallantoic fusion did not occur, and the placenta was absent or markedly decreased in size (Fig. 3g–j). Although most of the mutant embryos at 8.5 d.p.c. did not show obvious abnormalities, the mutants with the most marked placental abnormalities tended to be smaller and to have disorganized internal structure. By 9.5 d.p.c., the mutant placentas contained multiple layers of giant cells with no labyrinthine trophoblast or spongiotrophoblast (Fig. 3k–n). Mice derived from two independent targeted ES cell lines studied in C57BL, CD1 and 129/Sv genetic backgrounds displayed identical phenotypes (data not shown).

To confirm the histological findings in *Estrrb* mutant trophoblast tissues, expression of several markers of trophoblast differentiation

Table 1 Genotype of offspring from ERR- β heterozygous parents

Stage	Total	Genotype		
		+/+	+/-	-/-
E7.5	81	20	41	20
E8.5	134	33	64	37
E9.5	40	7	24	9*
E10.5	44	14	22	8*
E11.5	46	14	32	0
E12.5	10	3	7	0
Term	8	4	4	0
P28	54	19	35	0

E, embryonic day number; P, postnatal day number.

* Abnormal embryos.

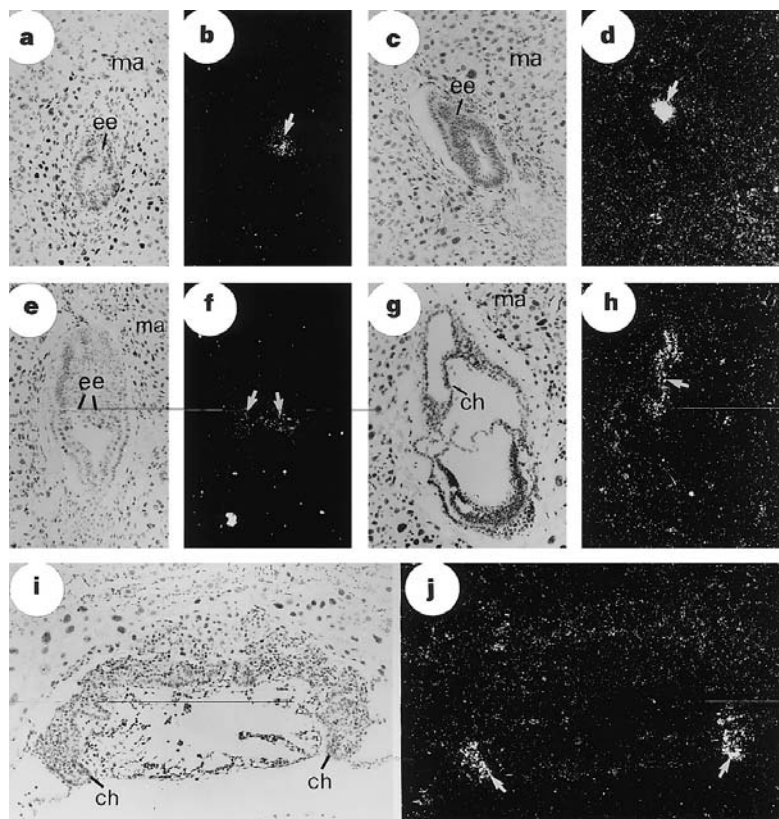


Figure 1 Expression of *Estrrb* in trophoblast tissues. Bright-field images of normal embryos at 5.5 (a), 6.0 (c), 6.5 (e), 7.5 (g) and 8.5 (i) d.p.c. RNA *in situ* hybridization was performed using a 320-bp riboprobe specific for *Estrrb* exon 2. *Estrrb* transcripts are present in the extra-embryonic ectoderm at 5.5 (b), 6.0 (d) and 6.5 (f) d.p.c. At 7.5 d.p.c., *Estrrb* transcripts are exclusively detected in chorion (h), and at 8.5 d.p.c. are present only at its free margin (j). *Estrrb* expression was not detected in the ectoplacental cone at any stage of development, nor was placental expression detected after 8.5 d.p.c. Arrows indicate regions of specific hybridization; ch, chorion; ee, extra-embryonic ectoderm; ma, maternal decidua.

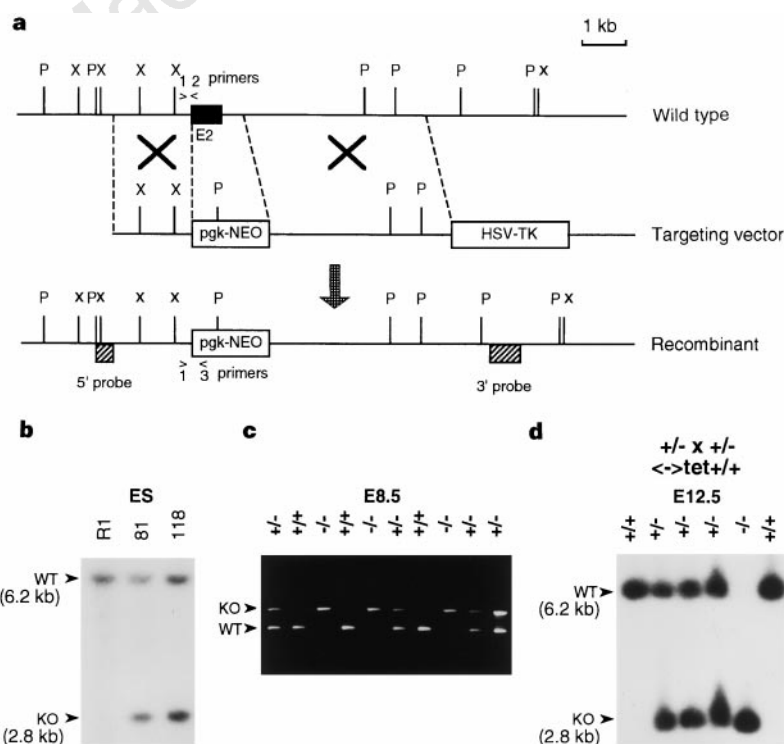


Figure 2 Targeted disruption of the *Estrrb* gene and heterozygote inbreeding analysis. **a**, Schematic representation of the *ERR-β* locus (wild type, top), targeting vector (middle), and targeted allele (recombinant, bottom). Arrows indicate the positions of primers P1, P2 and P3 used to genotype the offspring of heterozygous matings. Shaded rectangles represent the 5' and 3' probes used for Southern analysis of ES cell colonies. P, *Pst*I; X, *Xho*I. **b**, Southern blot analysis of targeted ES clones. DNA from parental ES cells (R1) and targeted clones (81 and 118) was digested with *Pst*I and hybridized to the 5' probe. The positions of bands corresponding to the wild-type allele (WT, 6.2 kb) and the mutant allele (KO, 2.8 kb) are indicated. **c**, PCR analysis of genotypes of embryos at 8.5 d.p.c. from a heterozygote intercross. **d**, *ERR-β* mutant embryos are present among the progeny at 12.5 d.p.c. of heterozygous intercross rescued by tetraploid aggregation.

was examined by RNA *in situ* hybridization. Expression of the *Mash-2* gene, which is a marker of spongiotrophoblast, labyrinthine trophoblast cells and their precursors³, was examined in *Estrrb* mutant embryos. At 7.5 d.p.c., *Mash-2* expression was not changed in the trophoblast region of *Estrrb*^{-/-} embryos (data not shown). Diploid trophoblast cells expressing the *Mash-2* gene were reduced in number at 8.5 d.p.c. (Fig. 4a–d) and absent at 9.5 d.p.c. in the *Estrrb*^{-/-} trophoblasts (Fig. 4g–j). Similar results were obtained with the markers 4311 (ref. 4) and Flt-1 (ref. 5), which have expression restricted to the spongiotrophoblast and its precursors (data not shown). In contrast, expression of transcripts for the trophoblast giant-cell markers placental lactogen-1 (ref. 6) and proliferin (ref. 7; data not shown) were markedly increased at both 8.5 d.p.c. (Fig. 4e, f) and 9.5 d.p.c. (Fig. 4k, l). To determine when diploid

trophoblast proliferation in *Estrrb* mutants became impaired, we examined the incorporation of 5-bromo-2'-deoxyuridine (BrdU) into DNA in trophoblast cells at different stages of development. At 7.5 d.p.c., the fraction of diploid trophoblast cells incorporating BrdU was similar in wild-type and mutant ectoplacental cones (approximately 85% of cells). However, at 8.0 and 8.5 d.p.c. the fraction of *Estrrb*^{-/-} diploid trophoblast cells incorporating BrdU was less than half that of normal trophoblast (between 30% and 40% of cells). Taken together, these results confirmed the histological observations and demonstrated that reduced proliferation of diploid trophoblast cells first occurred a few hours after failure of chorion development.

By 9.5 d.p.c., *Estrrb*^{-/-} embryos displayed impaired development, as would be expected if placental function were significantly

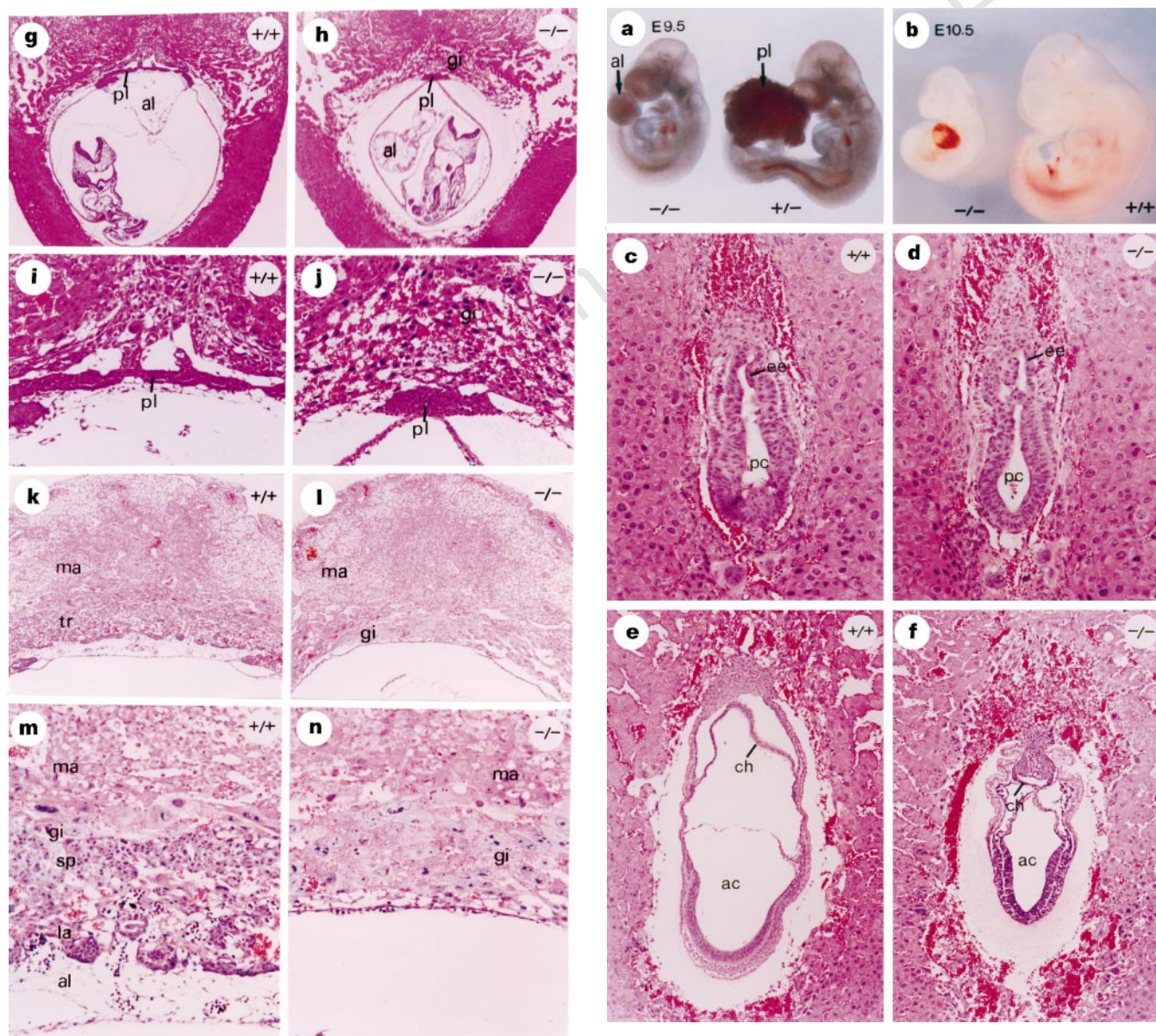


Figure 3 Morphology of homozygous mutant embryos and trophoblast tissues. **a, b**, Control and *Estrrb* mutant embryos at 9.5 (**a**) and 10.5 (**b**) d.p.c. At 9.6 d.p.c., the allantois of the *Estrrb* mutant has not fused to form the placenta. By 10.5 d.p.c., the *Estrrb* mutant embryo is dead and undergoing resorption. **c, d**, Wild-type (**c**) and mutant (**d**) embryos at 6.5 d.p.c. Development of the embryonic and extra-embryonic structures appear normal. **e, f**, Wild-type (**e**) and mutant (**f**) embryos at 7.5 d.p.c. The growth of the mutant embryo is retarded, and the chorion is diminished in size. **g, h**, Wild-type (**g**) and mutant (**h**) embryos at 8.5 d.p.c. The chorion and ectoplacenta of the mutant embryo are dramatically reduced in size with increased numbers of secondary giant cells. **i, j**, Higher power views of **g** and **h**, respectively. **k, l**, Wild-type (**k**) and mutant (**l**) trophoblast tissue at 9.5 d.p.c. In the mutant, diploid trophoblast cells are absent and secondary giant cells form multiple layers. **m, n**, Higher power views of **k** and **l**. Abbreviations: ac, amniotic cavity; al, allantois; ch, chorion; ee, extraembryonic ectoderm; gi, giant cells; la, labyrinthine trophoblast; ma, maternal decidua; pc, proamniotic cavity; pl, placenta; sp, spongiotrophoblast; tr, trophoblast.

impaired. Expression of ERR- β during early fetal development was present in specific regions of extra-embryonic ectoderm, although no ERR- β transcripts were detected in the embryo. This suggested that embryonic growth failure resulted from changes in placental function, a hypothesis that could be tested by performing tetraploid embryo rescue experiments^{8,9}. Diploid morulae from *Estrrb*^{+/-} intercrosses were aggregated with four-cell-stage wild-type tetraploid embryos, and the resulting chimaeras were transferred to CD1 foster mothers. In these experiments, wild-type tetraploid cells cannot contribute to embryonic cell lineages, but can contribute to trophoblast lineages, where they might compensate for defects present in the *Estrrb*^{-/-} offspring. Because *Estrrb*^{-/-} embryos die by 10.5 d.p.c., the pregnancies were allowed to continue to 12.5 d.p.c., when the embryos were genotyped (Fig. 2d). The offspring of four independent rescue experiments included eight *Estrrb*^{-/-} embryos (19% of the recovered embryos). In one experiment, the tetraploid component was marked by ubiquitous expression of the *Escherichia coli* β -galactosidase gene. This provided visual confirmation that all of the trophoblast cells were wild type, and therefore blue, in rescued -/- embryos. The *Estrrb*^{-/-} embryos were externally indistinguishable from their *Estrrb*^{+/+} littermates. These experiments indicate that the early embryonic growth retardation and lethality results solely from abnormal placental development in the *Estrrb*^{-/-} embryos.

We have shown that the nuclear hormone receptor ERR- β is essential for normal placental formation. Ablation of ERR- β in extra-embryonic ectoderm results in abnormal chorion formation followed by failure of diploid trophoblast self-renewal, increased formation of giant cells, placental failure and embryonic death. Impaired diploid trophoblast proliferation has been observed when ectoplacental cone fragments are cultured *in vitro*¹⁰ or grown ectopically¹¹. Trophoblast proliferation could be at least partly restored by transplanting ectoplacental cone fragments into the amniotic cavities of embryos at 7.5 d.p.c.; however, these growth conditions incompletely suppressed the formation of giant cells, suggesting that additional signals from extra-embryonic tissues were required for normal trophoblast development^{12,13}. ERR- β is expressed in only a subset of diploid trophoblast cells making up the chorion, and disruption of its function causes widespread failure of

diploid trophoblast proliferation, which occurs only after impaired formation of chorion. It is therefore possible that ERR- β mediates some critical function of the chorion in producing or modulating a diffusible signal that is necessary to support proliferation and block terminal differentiation of the diploid trophoblast. Common methods of influencing fertility have used drugs that target the oestrogen and progesterone receptors^{14,15}. As the classic targets of these hormonal agents do not seem to play a unique role in regulating implantation and placentation^{16,17}, identification of more specific means of influencing fertility could significantly increase the reproductive health of women. Pharmacological modulation of the activity of ERR- β , a nuclear hormone receptor that plays an essential role in early placentation, may provide means by which this may be accomplished. □

Methods

Targeted disruption of the *Estrrb* locus. The targeting vector was constructed using genomic DNA from a 129/Sv mouse strain. The 1.8-kilobase upstream region and the 4.1-kb downstream region of exon 2 were included in the targeting vector. The targeting vector replaces the first zinc-binding motif of the DNA-binding domain of the ERR- β protein with a *neo* cassette. The linearized construct was electroporated into R1 ES cells¹⁸, which were then selected with G418 (150 μ g ml⁻¹) and gancyclovir (2 μ M). Selected ES colonies were screened by probing *Pst*I-digested ES cell DNA with the 5' external probe, which hybridized to a 6.2-kb restriction fragment in the wild-type allele and 2.8-kb fragment in the knockout allele. We obtained 12 correctly targeted clones from 165 double-resistant colonies. Single integration of the targeting construct in these ES cell clones was confirmed by reprobing the Southern blots with a *neo* probe (data not shown). Appropriate integration of the 3' end of the targeting construct was verified using Southern blot analysis of *Xho*I-digested DNA using the 3' external probe (data not shown). Three clones were injected into CD1 blastocysts to generate chimaeras. Chimaeric males from two independent clones (81 and 118) passed the mutation to their offspring. PCR analysis of genotypes of embryos from a heterozygote intercross was performed using primers located in *Estrrb* intron 1 (P1, 5'-TCTTGCCATCCTTAGGCCAGC-3'), the 5' region of *Estrrb* exon 2 (P2, 5'-AACTAGAGCCCAGGTGCCTGT-3') and the *neo* cassette (P3, 5'-CCTCTGAGCCCAGAAAGCGAA-3'). DNA was obtained from embryonic tissue scraped from histological sections or from the yolk sac. Primers P1

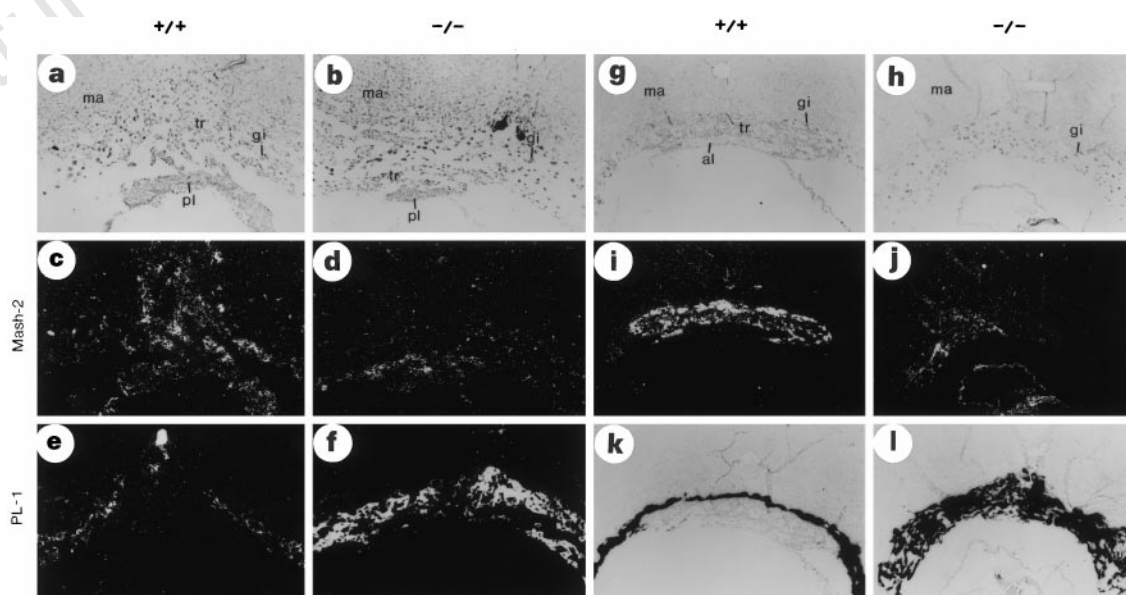


Figure 4 Expression of cell-type-specific markers in trophoblast tissues. Bright-field images of normal placentas at 8.5 (a) and 9.5 (g) d.p.c., and the corresponding sections for *Estrrb* mutants (b and h). RNA *in situ* hybridization was performed on near-adjacent sections using probes for Mash-2 (c, d, i and j) and PL-1 (e, f, k and l). Expression of the placental markers confirms a progressive reduction in the number of diploid trophoblast cells accompanied by a marked increase in the number of trophoblast giant cells between 8.5 and 9.5 d.p.c.. Abbreviations: al, allantois; gi, giant cells; ma, maternal decidua; pl, placenta; tr, trophoblast.

and P2 amplify a fragment of 291 base pairs from the wild-type allele; primers P1 and P3 amplify a product of 440 bp, indicating the presence of the targeted allele.

Histological and *in situ* hybridization studies. Embryos and trophoblast tissues from heterozygous intercrosses were fixed in 4% paraformaldehyde, dehydrated and embedded in paraffin. Sections (7 µm) were stained with haematoxylin and eosin¹⁹ or processed for *in situ* hybridization with radio-labelled riboprobes²⁰. Genotypes of embryos were identified by PCR using DNA from embryonic tissues scraped from histological sections.

BrdU labelling of trophoblast tissues. BrdU (100 µg per gram of body weight) was injected intraperitoneally into pregnant female mice. The mice were killed 1 h later, and the embryos and trophoblast tissues were removed and fixed in 4% paraformaldehyde and processed for immunohistochemistry. The genotype of each embryo was identified by PCR using DNA from embryonic tissues scraped from sections. Sections were incubated with an anti-BrdU monoclonal antibody²¹ (BROMO-2), and antibody binding was visualized with anti-mouse immunoglobulin-alkaline phosphatase (Boehringer Mannheim). Sections were stained with haematoxylin to visualize nuclei and allow cells to be counted.

Generation of tetraploid aggregation chimaeras. Electrofusion of two-cell-stage blastomeres collected from CD1 female mice or CD1 females crossed with male homozygous for the ROSA-26 gene trap insertion²² was used to produce wild-type tetraploid embryos. The fused embryos were cultured overnight in embryo culture medium in 5% CO₂ at 37 °C. Eight-cell-stage diploid embryos were recovered from the oviduct of embryonic-day (e)2.5 pregnant ERR-β heterozygote females mated to an ERR-β heterozygote male. Two four-cell tetraploid embryos were sandwiched around a diploid eight-cell-stage embryo from the heterozygous cross and aggregated overnight. Successfully aggregated embryos were transferred to e2.5-pseudopregnant CD1 recipients. Recipients were killed at e12.5 and embryos were genotyped and examined. Those aggregates made with ROSA-26 tetraploid embryos were also stained for β-galactosidase activity in the placenta, yolk sac and embryonic components²³.

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Inflammatory stimuli induce accumulation of MHC class II complexes on dendritic cells

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Dendritic cells have the remarkable property of presenting any incoming antigen¹. To do so they must not only capture antigens with high efficiency and broad specificity, but must also maximize their capacity to load class II molecules of the major histocompatibility complex (MHC) with antigenic peptides in order to present a large array of epitopes from different proteins, each at a sufficient copy number. Here we show that formation of peptide–MHC class II complexes is boosted by inflammatory stimuli that induce maturation of dendritic cells. In immature dendritic cells, class II molecules are rapidly internalized and recycled, turning over with a half-life of about 10 hours. Inflammatory stimuli induce a rapid and transient boost of class II synthesis, while the half-life of class II molecules increases to over 100 hours. These coordinated changes result in the rapid accumulation of a large number of long-lived peptide-loaded MHC class II molecules capable of stimulating T cells even after several days. The capacity of dendritic cells to load many antigenic peptides over a short period of initial exposure to inflammatory stimuli could favour presentation of infectious antigens.

A key feature of dendritic cells is that they can capture antigens in peripheral tissues and migrate to secondary lymphoid organs where they acquire the ability to stimulate naive T cells¹. This maturation process was originally noted in freshly isolated Langerhans cells which, upon culture *in vitro*, lost acidic organelles and the capacity to synthesize class II molecules and to present soluble antigen, while acquiring the ability to stimulate T cells^{1–6}. Although the final results of the maturation process is loss of antigen-capturing and -processing capacity, it is not known whether the generation of peptide–MHC class II complexes is enhanced during the transition from immature to mature dendritic cells.

We previously described an *in vitro* culture system suitable for analysing the maturation process of dendritic cells. Human monocytes cultured in medium supplemented with granulocyte-macrophage colony-stimulating factor (GM-CSF) and interleukin (IL)-4 develop into a homogeneous population with the characteristics of immature dendritic cells, namely high endocytic activity and a low capacity for stimulating T cells. When challenged by inflammatory stimuli such as tumour-necrosis factor (TNF)-α, IL-1 and lipopolysaccharide (LPS), these cells can no longer endocytose, they upregulate adhesion and co-stimulatory molecules, and become mature stimulatory dendritic cells^{7,8}. We used this system to study the effect of dendritic cell maturation on class II-restricted antigen presentation.

Cell-surface and total class II molecules were quantified in intact and permeabilized cells. As shown in Fig. 1a, immature dendritic cells express high levels of total class II molecules, but only a small fraction is present on the cell surface. During the first 24 hours after

stimulation with LPS or TNF- α , up to $\sim 6 \times 10^6$ class II molecules are deposited on the cell surface. This large increase is paralleled by an increase in total class II molecules, suggesting that both redistribution and new synthesis have occurred (Fig. 1b, c).

To investigate the large intracellular class II pool present in immature dendritic cells, we first measured class II internalization and recycling. In immature dendritic cells, biotinylated Fab fragments of an anti-DR antibody bound to surface class II molecules are rapidly internalized and recycled back to the cell surface (Fig. 2a). Within 5 min, about 50% of the Fab disappears from the cell surface, indicating that the internalization process is very rapid and that the recycling pool is as large as the surface pool. After stimulation with LPS or TNF- α , the large pool of recycling class II molecules progressively decreased and disappeared after 40 hours (Fig. 2b). This decrease was paralleled by a decrease in both fluid phase and receptor-mediated endocytic activity (Fig. 2c).

It has been previously shown that different antigenic peptides can be loaded either on newly synthesized⁹ or on recycling class II molecules^{10–12}. Given that in immature dendritic cells the pool of recycling class II molecules far exceeds that detected in other antigen-presenting cells (APC) such as B cells^{10,13,14}, we determined the relative contribution of recycling and newly synthesized class II molecules in antigen presentation. We pulsed immature dendritic cells at 37°C with a low concentration of tetanus toxoid (TT) and

measured the formation of specific peptide–class II complexes by following, as an early readout of T cell activation, the induction of Ca²⁺ flux in TT-specific T-cell clones¹⁵. As shown in Fig. 3, the epitope recognized by clone ALT220 was generated very rapidly (20 min after addition of TT), whereas the epitope recognized by clone ALT81 was generated only after 40–50 min. Blocking of protein synthesis by pretreating dendritic cells with cycloheximide did not affect presentation to clone ALT220, although it completely blocked presentation to clone ALT81, indicating that the former epitope (TT947–967) is loaded on preformed and recycling molecules, whereas the latter (TT924–938) is loaded on newly synthesized molecules. When Epstein–Barr virus (EBV)-B cells were used as APC, 20-fold higher concentrations of TT and longer incubation times (1 and 4 h for clones ALT220 and ALT81, respectively) were required (data not shown). These results indicate that immature dendritic cells can use both recycling as well as newly synthesized class II molecules for rapid and efficient presentation of incoming antigen.

Immature dendritic cells synthesize class II molecules at a high rate (3–5 times faster than EBV-B cells; data not shown) and this rate is further increased by maturation stimuli. As shown in Fig. 4a, a 2–3-fold increase in class II synthesis was observed as early as one hour after challenge with LPS or TNF- α and was sustained for 10–16 hours. But at later times, class II synthesis was completely shut off. MHC class I synthesis was also upregulated after maturation

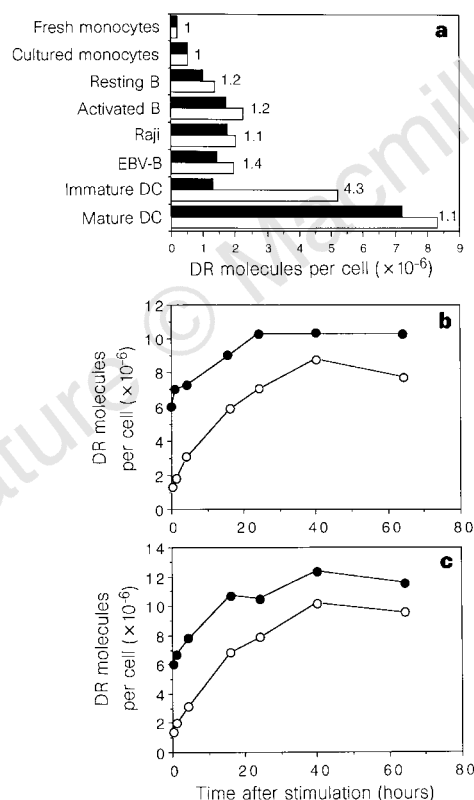


Figure 1 Increased expression of surface and total class II molecules in DC stimulated with LPS or TNF- α . **a**, Number of surface (black bars) and total (white bars) DR molecules in different APC. The number indicates to the total/surface ratio. **b**, **c**, Kinetics of increase in surface (○) and total (●) DR molecules in DC stimulated with LPS (**b**) or TNF- α (**c**). Results are representative of at least 5 different experiments.

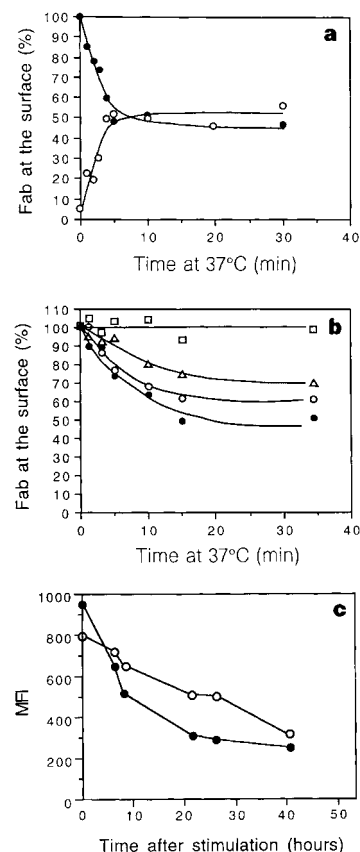


Figure 2 Extensive internalization and recycling of MHC class II molecules in immature DC is progressively lost together with endocytic activity following maturation. **a**, Internalization of surface-bound (●) and recycling of internalized (○) L243 biotinylated Fab fragments in immature DC. Recycling is calculated as per cent of internalized Fab¹⁰. Comparable results were obtained in three different experiments. **b**, Progressive loss of class II internalization in DC stimulated for 0 (●), 4 (○), 16 (△) and 40 h (□) with LPS. Comparable results were obtained using TNF- α . **c**, Accumulation of FITC-dextran (○) or Lucifer yellow (●) in DC stimulated with TNF- α . Comparable results were obtained using LPS. MFI, mean fluorescence intensity.

and was sustained for up to 64 hours (Fig. 4b). Total protein synthesis showed only small fluctuations (data not shown).

Our results indicate that in the first few hours after induction of maturation, dendritic cells are still able to internalize antigen at a high rate and have a high capacity for peptide loading as they still retain a large amount of recycling class II molecules while building up an increased pool of newly synthesized class II molecules. We therefore investigated whether inflammatory stimuli given together with the antigen could boost the formation of complexes between antigenic peptides and class II molecules. Immature dendritic cells were exposed to ^{125}I -labelled TT for 10 hours in the presence or absence of the maturation stimulus. Addition of LPS gave a 3–7-fold increase in the amount of SDS-stable class II dimers containing iodinated peptides (Fig. 5a, d). During the same period, total SDS-stable dimers only increased <2-fold (Fig. 5b–d). These results show that the maturation stimulus enhances the generation of antigenic complexes.

In the APC analysed so far, class II molecules and most peptide–class II complexes are very stable and turn over with the same half-life of ~30–40 hours^{16,17}. As this stability is critical to immunogenicity, we tested whether the maturation process could also affect class II half-life. Immature dendritic cells were pulse-labelled with ^{35}S -methionine/cysteine and chased in the presence or absence of LPS. As shown in Fig. 6a, b and e, newly synthesized class II molecules disappear rapidly in immature dendritic cells, with a half-life of ~10 hours (range, 8 to 18 hours in 5 experiments). Addition of LPS during the chase period increased class II half-life to more than 100 hours. In contrast, the half-life of class I molecules was not affected by maturation (Fig. 6f). Likewise, after cell-surface iodination, there was a comparable shift in the half-life of class II

molecules (Fig. 6c, d), indicating that the short half-life observed in immature dendritic cells is mainly due to degradation of class II molecules that had already reached the cell surface. We also measured the efficiency of transport in immature dendritic cells of newly synthesized class II molecules to the cell surface by combining metabolic labelling with surface biotinylation¹⁸. As shown in Fig. 6g, newly synthesized class II molecules are efficiently transported to the cell surface. By correcting for the amount of material loaded, the efficiency of biotinylation and the presence of the recycling pool, we estimate that >80% of newly synthesized class II molecules is delivered to the cell surface within 3 hours. Taken together with the functional data (Fig. 3), our results show that in immature dendritic cells newly synthesized class II molecules reach the cell surface loaded with antigenic peptides, but are subsequently degraded. Maturation results in a dramatic shift in class II half-life allowing accumulation and persistence of the complexes formed.

The stabilization of antigenic complexes following maturation was tested in a functional assay. As shown in Fig. 7, immature

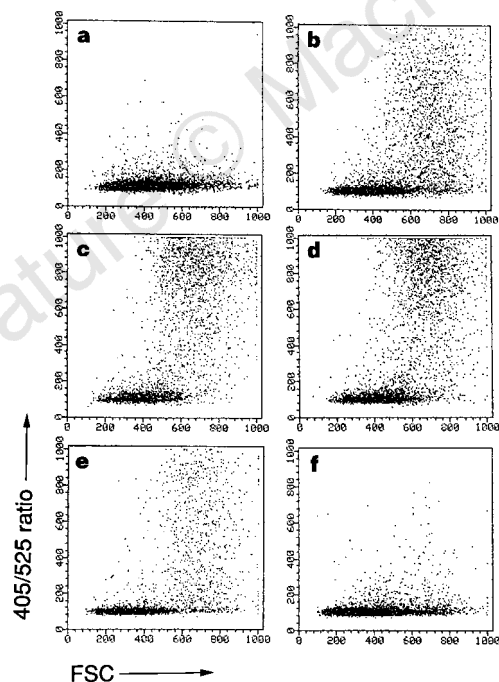


Figure 3 Immature DC can efficiently load antigenic peptides on both recycling and newly synthesized MHC class II molecules. Immature DC were pulsed with $2\ \mu\text{g ml}^{-1}$ TT and tested at different times for their ability to trigger an increase in $[\text{Ca}^{2+}]_i$ in T-cell clones ALT220, specific for TT947-967 (a–d) and ALT81, specific for TT924-938 (e, f). DC were either unpulsed (a) or pulsed with TT for 20 min (b), 40 min (c, d) or 60 min (e, f). In d and f, DC were pretreated for 7 h with $10\ \mu\text{g ml}^{-1}$ cycloheximide. The epitope recognized by clone ALT220 was generated in 15–20 min, whereas that recognized by clone ALT81 appeared after 40–50 min. When EBV-B cells were used as APC, the dose of antigen required was at least 20-fold higher and generation of the epitopes was delayed.

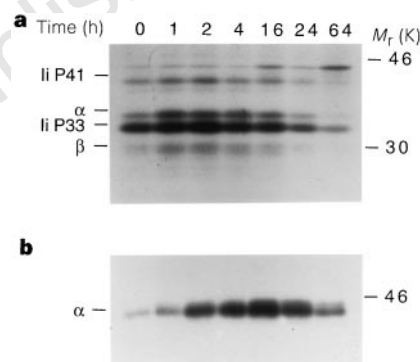


Figure 4 Transient upregulation of MHC class II synthesis in maturing DC. DC were metabolically labelled for 20 min before ($t = 0$) and at different times after LPS stimulation. **a**, MHC class II, and **b**, MHC class I immunoprecipitates separated by SDS-PAGE. The rate of increase in class II synthesis was 2–3-fold and comparable for six DC preparations stimulated by either LPS or TNF- α . Ii, invariant chain.

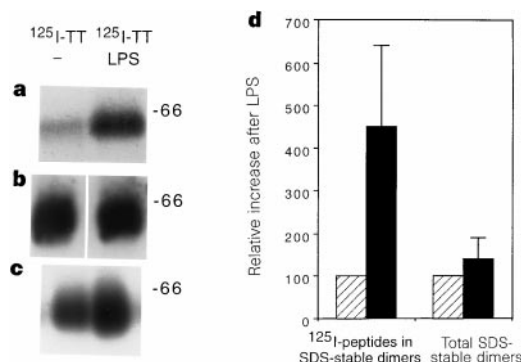


Figure 5 Maturation increases generation of SDS-stable class II dimers containing antigenic peptides. DC were incubated for 10 h with ^{125}I -labelled TT or unlabelled TT in the presence or absence of LPS. **a**, SDS-stable dimers containing iodinated peptides; **b**, total SDS-stable dimers measured in the same experiment by western blot developed with ^{125}I -labelled protein A; **c**, total SDS-stable dimers in immature and LPS-treated (10 h) DC detected by cell-surface iodination; **d**, quantification by Phosphorimager of ^{125}I -peptides in SDS-stable dimers (6 experiments), and of total SDS stable dimers (3 experiments) in untreated DC (hatched bars) and LPS-treated DC (black bars).

dendritic cells can efficiently stimulate T cells if tested immediately after pulsing with antigen, but lose this capacity over time. However, when LPS was added during the chase period, the same cells could still strongly stimulate T cells after 72 hours. Together these results indicate that antigenic memory is induced by the maturation process.

We have shown that different mechanisms coordinate to maximize the antigen-presenting capacity of dendritic cells over a short period following exposure to inflammatory stimuli. In the first 24 hours after maturation is induced, antigenic peptides are efficiently loaded onto the rapidly recycling class II pool and onto newly synthesized class II molecules, which are produced in increased amounts. There is a key shift in class II half-life, which, together with the increased rate of synthesis, results in the rapid accumulation of a

large number of stable class II complexes that can be displayed for long periods, enabling dendritic cells to maintain long-term memory of past encounters with antigen.

The degradation of class II molecules in immature dendritic cells and their stabilization after maturation occur in both human and mouse. In murine 'early' dendritic cells, newly synthesized class II molecules are transported inefficiently to the cell surface and degraded, whereas in human immature dendritic cells the transport to the cell surface is relatively efficient and molecules that have already reached the plasma membrane are rapidly degraded. One explanation may be that degradation of class II molecules in human dendritic cells, is linked to their high rate of endocytosis⁸. A half-life of ~10 hours could be explained by the failure of as few as 0.5% of internalized class II molecules to recycle. In mature dendritic cells,

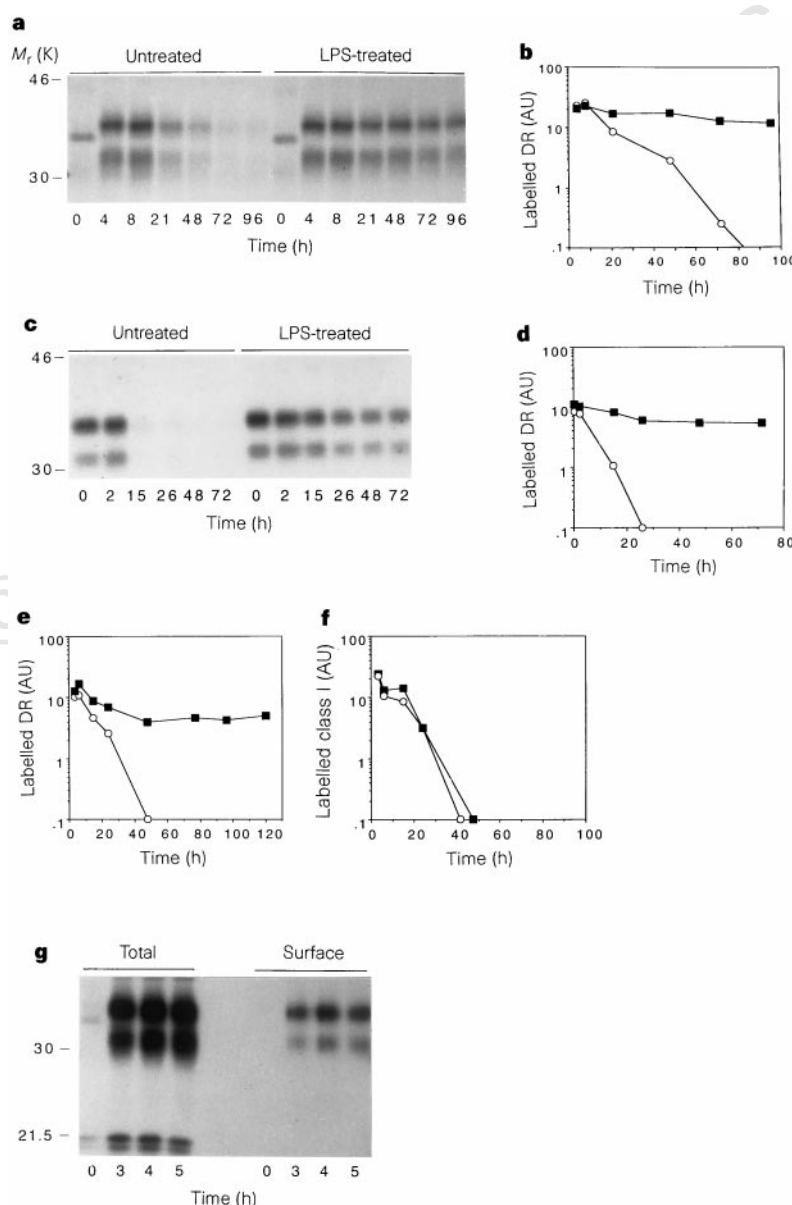


Figure 6 Maturation stimuli increase the half-life of class II molecules from ~10 to >100 hours. **a**, Stability of biosynthetically labelled class II molecules in the absence or in the presence of LPS, and **b**, quantification by Phosphorimager of the radioactivity associated with the α -chains in immature DC (○) or in DC chased in the presence of LPS (■) AU, arbitrary units. **c**, Stability of surface-iodinated class II molecules in the absence or in the presence of LPS, and **d**, quantification. **e**, **f**, Stability of biosynthetically labelled class II molecules (**e**) and class I molecules (**f**) measured in the same experiment. **g**, Efficient transport of newly synthesized class II molecules to the cell surface in immature DC. DC were biosynthetically labelled and surface-biotinylated after 0, 3, 4 and 5 hours of chase. The class II immunoprecipitates were analysed as such (left) or after reprecipitation with streptavidin-Sepharose (right). The amount of class II molecules delivered to the cell surface (>80% in 3 h) was calculated as described¹⁸.

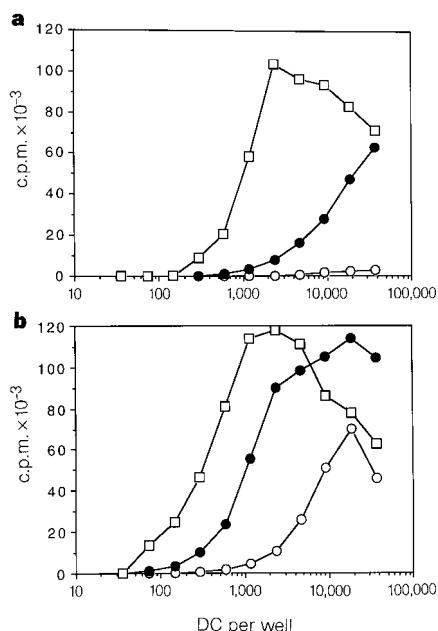


Figure 7 Maturation allows DC to maintain memory of antigens encountered. Immature DC were pulsed with **a**, 5 µg ml⁻¹ TT, or **b**, 0.1 µM TT830-843 peptide for 3 h at 37°C, washed and either tested immediately (□), or re-cultured in the presence (●) or absence (○) of LPS for 72 h before being tested for their ability to stimulate a specific T-cell clone KS140. Note that the clone does not express CD28 and is thus insensitive to co-stimulation.

decreased endocytosis may account for the very long half-life (over 100 hours) which exceeds that estimated in other cell types^{16,17}. Class II internalization and recycling may thus have the dual function of allowing rapid loading of peptides and of shortening the half-life of preformed molecules, so preventing the accumulation of complexes before encounter with the inflammatory stimulus. The shift in half-life of class II molecules in human and murine dendritic cells ensures that only those epitopes generated at sites of inflammation will be displayed in lymphoid organs for T-cell priming. □

Methods

Dendritic cells. Immature dendritic cells (DC) were prepared as described^{7,8}. Peripheral blood monocytes purified by centrifugal elutriation were cultured in RPMI-1640 supplemented with 10% FCS, 50 ng ml⁻¹ GM-CSF and 1,000 U ml⁻¹ IL-4 for 6–8 days. Maturation was induced by addition of either 1 µg ml⁻¹ LPS (from *S. abortus equi*, Sebak) or 50 ng ml⁻¹ recombinant TNF-α (R&D Systems). Uptake and accumulation of FITC-dextran or Lucifer yellow (Molecular Probes) have been described⁸.

Immunofluorescence. Cells were fixed for 30 min with 2% paraformaldehyde and then either permeabilized for 30 min with PBS containing 0.5% saponin, 5% FCS and 10 mM HEPES, or stained. Cells were stained with saturating concentrations of L243 anti-DR antibody (ATCC), followed by FITC-labelled goat anti-mouse IgG2a (Southern Biotechnology Associates). The number of DR molecules was calculated by extrapolation of the calibration curve obtained using beads coated with known amounts of mouse IgG2a (Qifkit, DAKO).

Internalization and recycling of class II molecules. Fab fragments of L243 antibody were biotinylated with NHS-SS-biotin (Pierce)¹⁰. DC were incubated at 0°C for 2 h with 30 µg ml⁻¹ Fab, washed in cold medium, shifted at 37°C for various times and rapidly transferred to cold medium. The amount of Fab remaining on the cell surface was determined using ¹²⁵I-labelled streptavidin (Amersham) as described¹⁰. To detect recycling, bound Fab were allowed to equilibrate for 20 min at 37°C. Cells were chilled and surface biotin was removed using two rounds of treatment with 50 mM glutathione (Sigma). The cells were then shifted to 37°C for various times and the biotinylated Fab reappearing on the cell surface detected as described.

Class II synthetic rate. At different times after stimulation with TNF-α or LPS, DC were labelled with ³⁵S-methionine/cysteine (Amersham) for 20 min.

Cells were lysed and MHC class II molecules immunoprecipitated using a polyclonal anti-class II rabbit antiserum (from H. Ploegh) and protein A-Sepharose.

Loading of antigenic peptides on SDS-stable class II dimers. TT (Connaugh) was iodinated to a specific activity of 5 × 10⁴ c.p.m. per ng with ¹²⁵I using the iodogen method (Pierce)⁹. 3 × 10⁶ DC were incubated with ¹²⁵I-TT (3 × 10⁸ c.p.m.) in the absence or presence of LPS. After 10 h, the cells were washed and lysed and class II molecules were precipitated with DA6.231 (anti-DR) antibody. Class II molecules were eluted at 24°C with 2% SDS buffer under non-reducing conditions and analysed by SDS-PAGE¹⁹. Replica gels were transferred to nitrocellulose and total levels of class II molecules were measured using a rabbit anti-class II antiserum followed by ¹²⁵I-protein A (Amersham) and quantified by using a Phosphorimager.

Half-life of class II molecules. Immature DC were either biosynthetically labelled with ³⁵S-methionine/cysteine for 20 min or surface-iodinated by the SHPP method¹³ and cultured in the presence or absence of LPS. After various times of chase, the same number of cells were lysed and MHC class II molecules were precipitated with L243 mAb and analysed by SDS-PAGE under reducing conditions. The radioactivity in specific bands was quantified by Phosphorimager. DC do not divide during the assay.

Transport of newly synthesized class II molecules to the cell surface. This assay was done as described¹⁸. Immature DC were labelled with ³⁵S-methionine/cysteine for 20 min and chased in normal medium. At 0, 3, 4 and 5 h, cells were surface-biotinylated using 2 mg ml⁻¹ NHS-SS-biotin (Pierce) on ice for 11 min. After washing, the cells were lysed and class II molecules were immunoprecipitated with L243 mAb. Precipitates were boiled in 100 µl PBS 2% SDS. 30 µl were analysed directly (total class II) and 70 µl were diluted into 1 ml PBS 2% Triton X100 and reprecipitated with streptavidin-Sepharose (Zymed) (surface biotinylated class II). The efficiency of transport to the cell surface was calculated¹⁸ by considering the amount of material loaded, the efficiency of biotinylation (~30%) and the presence of an internalized pool of class II molecules.

Antigen-presentation assay. To measure the kinetics of antigen processing¹⁵, DC were incubated with 2 µg ml⁻¹ TT at 37°C and tested at different times for their ability to trigger an increase in [Ca²⁺] in TT-specific T-cell clones loaded with Indo1 AM (Sigma). Cycloheximide (10 µg ml⁻¹; Sigma) was added to DC 7 hours before the assay. To estimate the persistence of specific peptide-MHC complexes, DC were pulsed with TT protein or TT830-843 peptide and cultured in the presence or absence of LPS for 72 h. Graded numbers of cells were tested for their capacity to trigger T-cell proliferation of a specific T-cell clone. Clones used in combination with autologous or class II-matched DC were ALT220 (specific for TT947-967), ALT81 (TT924-938) and KS140 (TT830-843).

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Developmental regulation of MHC class II transport in mouse dendritic cells

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Dendritic cells (DCs) have the unique capacity to initiate primary and secondary immune responses^{1–3}. They acquire antigens in peripheral tissues and migrate to lymphoid organs where they present processed peptides to T cells. DCs must therefore exist in distinct functional states, an idea that is supported by observations that they downregulate endocytosis and upregulate surface molecules of the class II major histocompatibility complex (MHC) upon maturation^{4–7}. Here we investigate the features of DC maturation by reconstituting the terminal differentiation of mouse DCs *in vitro* and *in situ*. We find that early DCs, corresponding to those found in peripheral tissues, exhibit a phenotype in which most class II molecules are intracellular and localized to lysosomes. Upon maturation, these cells give rise to a new intermediate phenotype in which intracellular class II molecules are found in peripheral non-lysosomal vesicles, similar to the specialized CIIV population seen in B cells. The intermediate cells then differentiate into late DCs which express almost all of their class II molecules on the plasma membrane. These variations in class II compartmentalization are accompanied by dramatic alterations in the intracellular transport of the new class II molecules and in antigen presentation. We found that although early DCs could not present antigen immediately after uptake, efficient presentation of the previously internalized antigen occurred after maturation, 24–48 hours later. By regulating class II transport and compartmentalization, DCs are able to delay antigen display, a property crucial to their role in immune surveillance.

Mouse bone marrow is a major source of DCs when cultivated with granulocyte-macrophage colony-stimulating factor (GM-CSF)⁸. Immunofluorescence microscopy of these cultures revealed three distinct developmental stages. Cells were identified as DCs by the expected repertoire of antigens and expression of MHC class II, cell shape, and adherence. As reported previously⁸, DCs were found

by immunofluorescence or FACS to be negative or weakly positive for the granulocyte marker GR1, negative for the macrophage marker SER-4, but strongly positive for CD11c and MHC class II. Contaminating SER-4 or GR1-positive cells were negative for class II and judged not to be DCs.

After 4–5 days, DCs were found in proliferating clusters loosely attached to adherent stromal cells⁸. By confocal microscopy, most of the cells present in or migrating out from the clusters showed little MHC class II on their surface, but contained abundant intracellular class II (Fig. 1a). The class II-positive vesicles represented lysosomes (MIICs) and late endosomes, being positive for IgB-B/lamp-2 and H2-M (Fig. 1a). Thus, they were characteristic of MIIC as defined in human lymphoblasts and human DCs^{9–12}. As the MIIC-containing cells were present in proliferating clusters, we defined them as ‘early’ DCs.

With increasing time in culture, two additional cell populations were detected. The first of these (‘intermediate’ DCs) was present transiently and comprised non-adherent cells that had little surface MHC class II (Fig. 1b). They were strikingly unlike the early cells, however, because most of their intracellular class II was in a vesicle population that was devoid of lysosomal markers (Fig. 1b, arrows), and thus reminiscent of non-lysosomal, class II-positive CIIV isolated from A20 B cells¹³. At later times in particular, the vesicles accumulated directly beneath the plasma membrane (Fig. 1b, right), whereas the lysosomes became concentrated in the perinuclear region.

The third major DC population accumulated with time until by 8–10 days it represented almost all of the non-adherent class II-positive cells. These ‘late’ cells had a more classical DC phenotype, with long processes that stained for class II (green) (Fig. 1c). Little class II remained intracellularly, with most of the now largely class II-depleted H2-M/lamp-2-positive lysosomes visualized as poorly resolved clusters of red-staining vesicles in the perinuclear region. Further characterization indicated that markers such as DEC-205 and 2A1 were absent from early cells but expressed at moderate and high levels on intermediate and late cells, respectively^{8,14} (results not shown). Early cells, but not late cells, were capable of efficient fluid endocytosis (W. Garrett and I.M., unpublished results), as found previously for human cells⁴.

To determine whether early cells pass through the intermediate phenotype before reaching maturity, we produced highly purified populations of early cells by gently dislodging and isolating proliferating clusters on serum columns, followed by depletion of contaminating cells by fluorescent-activated cell sorting (FACS)⁸. This approach yielded ~95% pure populations of early DCs. As quantified in Fig. 1d, after 5–8 h in culture, the early cells had nearly disappeared and >60% of the population of exhibited the intermediate phenotype (for example, see Fig. 1b); 20–30% exhibited the late or mature phenotype (as shown in Fig. 1c). After 24 h, ~90% of the cells were found to be of the late phenotype. As the number of cells remained constant throughout, these results strongly suggest that there is a sequential relationship in the maturation pathway. The rapid maturation kinetics observed using purified cluster-derived cells probably reflect their greater developmental synchrony.

To ensure that the developmental sequence was not peculiar to bone marrow cultures, we investigated whether tissue DCs had similar properties. We first examined epidermal Langerhans cells¹⁵. In epidermal explants, class II was present in these cells in a punctuate pattern which co-localized with lysosomal marker H2-M, reminiscent of early bone marrow DCs (Fig. 2, upper right panels). If explants were incubated in culture medium and the Langerhans cells allowed to mature *in situ*, within 4 h the degree of class II and H2-M co-localization decreased, with class II staining remaining punctuate but becoming progressively less coincident with H2-M, which became concentrated at the cell body (Fig. 2, right panels). This pattern was consistent with the intermediate

phenotype. By 8 h, class II staining appeared homogeneous over the entirety of the cell, suggesting that it was present on the plasma membrane (fig. 2, lower right panels), consistent with cells of the late phenotype.

These results were confirmed by monitoring the development of Langerhans cells isolated from the explants¹⁶, which were found to follow the same morphological transitions observed using the bone marrow cultures (Fig. 2, left panels). Similar results were obtained for splenic DCs derived from red pulp (not shown). Thus, DCs in peripheral tissues adopt the early phenotype but can be induced by isolation, cytokine exposure or physical disruption to undergo the same type of maturational pattern as DCs in bone marrow culture.

The shift of MHC class II from lysosomal to non-lysosomal structures was confirmed by immuno-electron microscopy and by cell fractionation in Percoll density gradients; MHC class II $\alpha\beta$ dimers in lysosomal and non-lysosomal fractions alike were found in the SDS-stable conformation, suggesting that they were loaded with peptide (not shown). Of greater interest was to determine

whether the sequential appearance of class II in lysosomal then non-lysosomal structures reflected a shift of intracellular class II from lysosome-like MIIC to endosome-like class II vesicles (CIIV), similar those found in murine B cells¹³. CIIVs represent a distinct vesicle population and are resolved from conventional endocytic organelles by free-flow electrophoresis¹⁷. We fractionated early and late DC populations by free-flow electrophoresis and found, as expected, that each population had a typical fractionation pattern: plasma membrane markers (LFA-1 detected by western blot) were restricted to unshifted fractions containing the major peak of cell proteins, whereas endosome- and lysosome-containing fractions (detected by β -hexosaminidase) were deflected towards the anode (results not shown).

When the free-flow electrophoresis fractions were probed by western blotting (Fig. 3a), early DCs exhibited class II in unshifted fractions (containing endoplasmic reticulum, Golgi and plasma membranes), as well as in fractions that co-migrated with lysosomal markers. The pattern was similar for cultures containing intermedi-

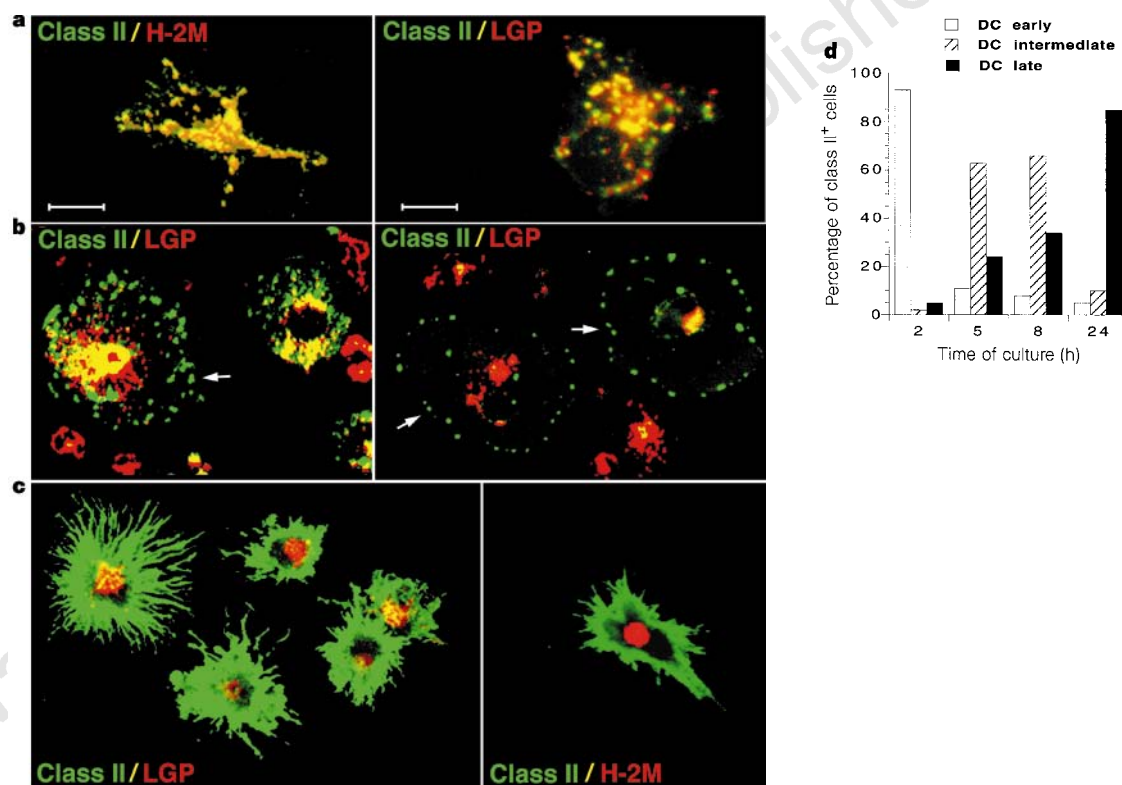


Figure 1 MHC class II distribution defines three distinct stages in the developmental maturation of bone-marrow-derived dendritic cells *in vitro*. DCs from bone marrow cultures were collected, plated on glass coverslips, and processed for immunofluorescence and confocal microscopy. In each case, MHC class II molecules were visualized using an FITC-conjugated second antibody and displayed as green staining. **a**, Early-DC phenotype. Optically merged confocal image of cells derived from proliferating clusters and stained for H2-M (red, left) or lysosomal membrane protein lgp-B/lamp-2 (LGP, red; right) and class II (green). Cells were isolated on day 4 of culture in the presence of GM-CSF. Class II co-localizes with both H2-M and lgp/lamp (yellow). Scale bars, 10 μ m. **b**, Intermediate-DC phenotype. Optically merged confocal image of non-adherent cells stained for lgp-B/lamp-2 (red) and class II (green) after 6–7 days of culture in the presence of GM-CSF. Arrows indicate the presence of class II-containing vesicles depleted of lysosomal markers. Cells in the images on the left in the panels are the first to appear in culture and still contain some co-localized class II and lysosomal markers in the perinuclear region. Cells in the images on the right appear later and show a typical alignment of the non-lysosomal CIIV-like structures just beneath the plasma membrane. The CIIV-like structures were all unlikely to be macropinosomes as they were negative for surface markers MHC class I and LFA-1, both of which gave circumferential staining (not shown). Class II-negative cells staining for lgp/lamp in the field represent granulocyte or monocyte contaminants of the culture. **c**, Late DC phenotype. Optically merged confocal image of non-adherent DC stained for lgp-B/lamp-2 (left, red) or H2-M (right red) and class II (green) on day 8 of culture. All cells have a dendritic, sea-urchin-like appearance, with most class II molecules now on the cell surface. Lysosomes often appeared as a poorly resolved cluster concentrated in the perinuclear cytoplasm and devoid of MHC class II. **d**, Sequential appearance of early, intermediate and late DC phenotypes in highly purified cultures. Proliferating clusters were isolated on 50%-serum columns and early DCs further purified by depletion of GR1-positive granulocyte by FACS. Nearly homogeneous populations of early cells (95% pure) were returned to culture in the presence of GM-CSF and assayed by immunofluorescence microscopy at after 2, 5, 8 and 24 h. The total number of cells was constant at each time point, indicating that there was neither cell loss nor proliferation. Using the phenotypic criteria defined for **a–c**, the cell distribution in each population was quantified. After 5 h, only 12% of the cells were of the early phenotype, whereas >60% were of the intermediate phenotype; late cells had increased to represent >20% of the population. By 24 h, few early or intermediate cells remained and cultures consisted mainly of late cells.

ate and late cells but, in addition, a distinct peak of class II was detected in strongly anodally shifted membranes whose electrophoretic migration was identical to CIIV. Although it needs to be confirmed that these anodally shifted class II-containing membranes are functionally analogous to B-cell CIIV, it is interesting that a similar population of vesicles can be detected in developing DCs with non-lysosomal class II-containing vesicles.

To determine whether DC maturation is accompanied by altered transport of MHC class II molecules, we did pulse-chase experiments using cluster-derived early DCs or cultures containing intermediate and late cells. We first showed by metabolic labelling that the relative rates of class II synthesis were similar in the two cell populations, with late cells synthesizing ~20% more class II per hour than early cells (results not shown).

Next, early and late DCs were pulse-labelled for 20 min and the arrival of class II molecules at the plasma membrane monitored by cell-surface biotinylation^{18,19}. As shown in Fig. 3b, cell-surface delivery was strikingly inefficient in early DCs: although Ii chain-free $\alpha\beta$ dimers could be detected in total lysates within 60 min of chase, only a small fraction of these were inserted into the cell surface after 240 min (Fig. 3b, top panels). In contrast, late DCs were much better at delivering labelled class II molecules to the surface (Fig. 3b, middle panels): by 4 h, a large fraction of labelled $\alpha\beta$ dimers had reached the cell surface. This efficient delivery in late DCs was comparable to that in murine A20 cells.

The results from several pulse-chase experiments were quantified

by phosphorimaging to determine the percentage of total precipitable class II recovered after biotinylation. The results revealed that late DCs were at least 10-fold more efficient at transporting $\alpha\beta$ dimers to the surface than were early cluster-derived cells (Fig. 3c). After correcting for the inherent inefficiency of the biotinylation procedure (see Methods)¹³, we estimate that late DCs inserted at least 50% of their newly synthesized class II molecules into the plasma membrane, as compared to <5% for early cells and almost 100% for A20 B cells.

As early DCs localize most of their class II to MIIC, newly synthesized $\alpha\beta$ dimers are probably targeted to lysosomes and therefore have a different fate from those that reach the surface of late cells. In both early and late cells, peptide loading (as judged by stability in SDS) was the same (Fig. 3d), but class II molecules in early cells were degraded more rapidly than in late cells (in early cells the half-life was ~12 h, as compared to 36–40 h in late cell populations; Fig. 3e). Nevertheless, some of the class II synthesized in early cells probably did survive to reach the plasma membrane. In pulse-chase experiments done over 24 h, about 15% of the class II labelled during an initial 20 min pulse became accessible to surface biotinylation after 18 h (results not shown).

Our results are consistent with the belief that DCs accumulate antigen in the periphery and deliver it to lymphoid organs, and they indicate that peripheral early cells may sequester class II–peptide complexes intracellularly to prevent ‘premature’ presentation to T cells in the periphery. To test this, we incubated cluster-derived early

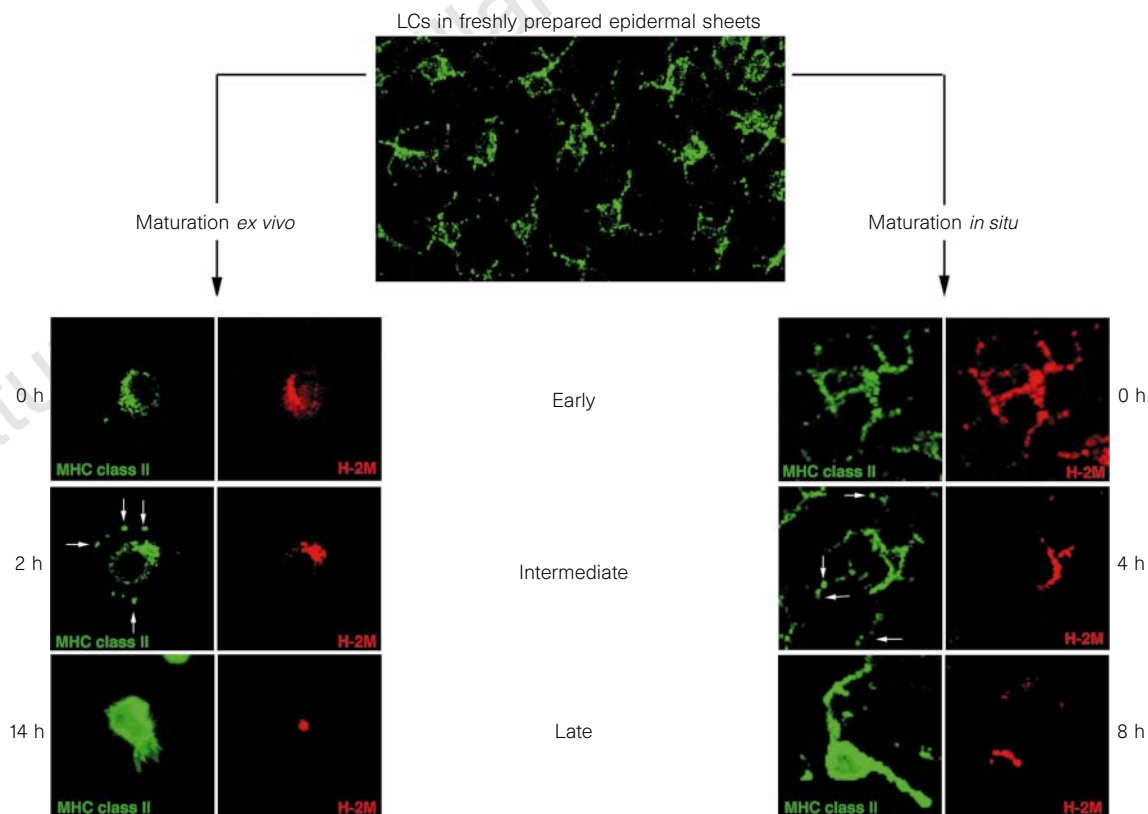


Figure 2 Developmental maturation of Langerhans cells (LCs) in culture and *in situ*. Epidermal sheets were cultured *in situ* or used for the isolation of individual LCs following collagenase treatment^{15,16}. Both explants and single cells were stained for MHC class II (using the mAb M5/114) and H-2M (using an affinity-purified rabbit antibody against the H-2M cytoplasmic domain) and visualized by confocal microscopy. Top, a low-magnification view of MHC class II-positive LCs in an intact epidermal sheet. Class II staining gives a punctate appearance in cells with typically dendritic LC morphology; cells appear to form a network throughout the epidermis. Right, higher-magnification views of LCs in epidermal sheets cultured *in situ* for 0, 4 or 8 h, and then stained for class II (green) and H-2M to mark lysosomes (red). Co-localization was strong directly after explant between class II and H-2M. Co-localization decreased with time, with H-2M staining become concentrated in the cell body while class II staining extended throughout each cell, presumably at the plasma membrane. Left, maturation of single LCs cultured for 0, 2 or 14 h *ex vivo*, that is, after isolation from an epidermal sheet. Cells were stained for class II (green) and H-2M (red) and had the morphological and organizational features of early, intermediate and late DCs from bone marrow cultures.

cells with various concentrations of ovalbumin for 3 h, washed them, and then cultured them for 0, 24 or 48 h without antigen. The cells were fixed to arrest development and assayed for immunogenic complexes. There was little presentation in the early cells fixed directly after the 3 h antigen uptake, even at very high antigen concentrations (Fig. 4). However, after incubation for 24–48 h in the absence of antigen, presentation activity increased dramatically, with virtually all of the cells achieving the mature phenotype by 48 h. Thus, immature DCs, similar to cells found in peripheral tissues, can accumulate antigen for later presentation.

A different picture emerged when the same experiment was done with the A20 B-cell line. Although widely used as an efficient antigen-presenting cell (APC), exposure of A20 cells to ovalbumin for 3 h resulted in relatively little presentation when fixed cells were assayed either before or after the chase (Fig. 4). Although our

concentrations of antigen were high, most studies with A20 cells or DCs have used 1–3-day incubations with antigen (often receptor-bound) and incubation of unfixed APCs with T cells, rather than the pulse-chase–fixation protocol used here. In control experiments, presentation was maximal for both DCs and A20 cells at >100-fold lower antigen concentrations if cells were incubated with ovalbumin for 48 h and unfixed cells were used for T-cell assays.

Early DCs in the periphery should thus accumulate antigen and newly synthesized class II molecules in lysosomes or MIIC, where processing and peptide loading can occur and the resulting immunogenic complexes can be retained. Although many of these complexes are degraded, some may survive until the DCs start maturing and class II–peptide complexes can be transferred to the plasma membrane, perhaps via the CIIV-like structures seen in the intermediate DC population. Alternatively, if only a few of the

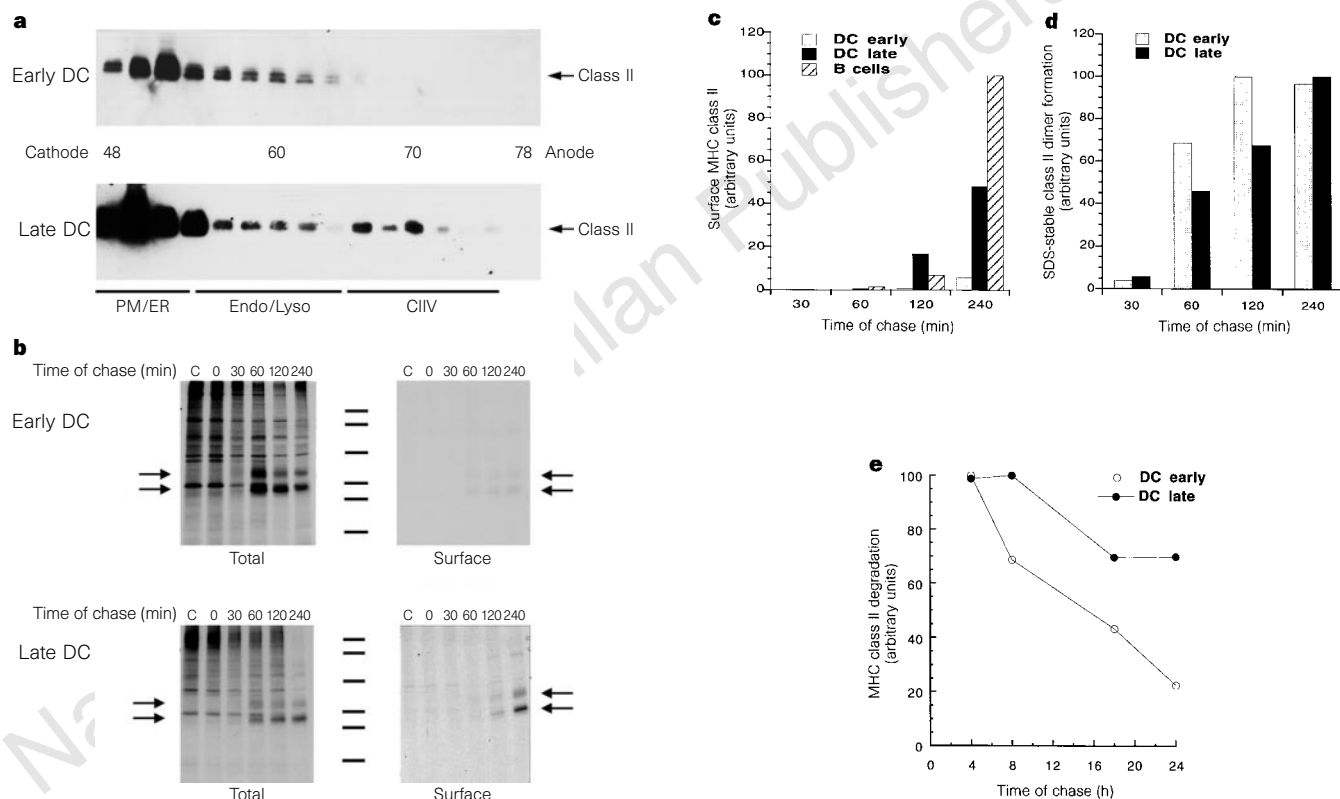


Figure 3 Intracellular transport of newly synthesized MHC class II is tightly regulated during the maturation of dendritic cells. **a**, Free-flow electrophoresis. Western blots for MHC class II β -chain corresponding to the separation by free-flow electrophoresis of intracellular membranes in early (top) or intermediate and late DCs (bottom). The separation profiles of early and late cells were virtually identical. Lysosomes (β -hexosaminidase; peak at around fraction 60) were anodally shifted relative to the plasma membrane (LFA-1; peak at around fraction 50) by at least 10 fractions (not shown). In early DCs, class II molecules were detected in the fractions corresponding to the plasma membrane (PM) (which also contained endoplasmic reticulum (ER); not shown) as well as in fractions containing endosomes and lysosomes. In intermediate and late DC populations, class II was detected in the PM/ER fractions, as well as in the endosome and lysosome fractions. A distinct peak of class II was anodally deflected relative to the major lysosome peak; this electrophoretic mobility corresponded to that of CIIV¹³. As already observed by immunofluorescence, the amount of class II present in PM-containing fractions of late DCs was much higher than for early DCs. **b, c**, Detection of class II in total cell lysates and on the cell surface. DCs and A20 B cells were pulse-labelled for 20 min and chased for the indicated times before cell-surface biotinylation. MHC class II molecules were immunoprecipitated using Y3P mAb and the surface appearance was determined by streptavidin-agarose precipitation of the biotinylated class II molecules. In total cell lysates, labelled class II became accessible to precipitation after 30 min, as expected because Y3P only detects class II molecules after $\alpha\beta$ chain dissociation²⁵. In early DCs, no class II was detected at the cell surface even after 240 min of chase. In late DCs, significant amounts of labelled class II became biotinylated at the surface after a 120-min lag, as in A20 B cells that transport nearly 100% of their class II to the surface^{13,24}. Quantification of two experiments is shown in **c**. The ratio of surface class II to total class II was corrected for biotinylation efficiency (see Methods) and expressed in arbitrary units (sample variations <20%). After 4 h, late DCs had 10-fold more class II at the surface than did early DCs, despite the fact that class II synthesis in late DCs was <20% higher than in the early cells. The total amount of class II did not change over this time, as detected by western blot. **d**, SDS-stable dimer formation was comparable in early and late DCs. Formation of SDS-stable $\alpha\beta$ dimers in early versus late DCs was determined in total cell lysates using unboiled samples for SDS-PAGE. The 65 kD SDS-stable dimer was quantified by phosphorimaging and plotted in arbitrary units. **e**, Turnover of MHC class II molecules in early and late DCs. DC cultures were labelled with ³⁵S-Translabel for 1 h and then chased for the indicated times. Total class II was immunoprecipitated using M5/114 mAb, boiled samples were analysed by SDS-PAGE, and α - or β -chain quantified by phosphorimaging. Class II half-lives were determined by linear regression to be ~12 h for early cells and 35–40 h for late cells.

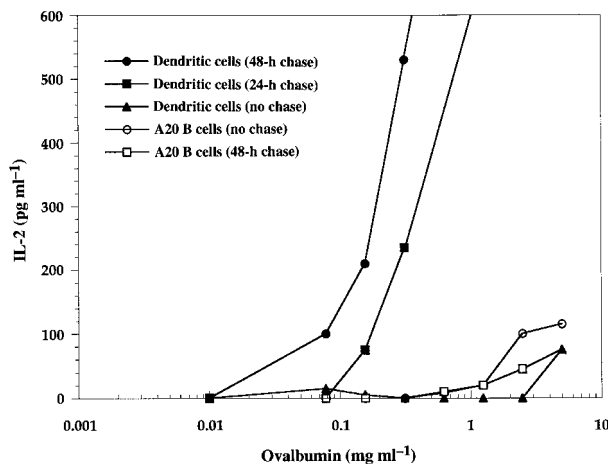


Figure 4 Early dendritic cells can accumulate antigen for presentation at a later time. Cluster-derived early DCs or A20 B cells were collected, incubated with the indicated concentrations of ovalbumin for 3 h, washed extensively, and then replated in ovalbumin-free medium for 0, 24 or 48 h before fixation and incubation with the ovalbumin-specific T-cell hybridoma DO.11. T-cell assays involved 1×10^4 DCs or 1×10^5 A20 cells incubated with 1×10^5 DO.11 cells for 24 h in 96-well plates as described²⁶. Stimulation was determined by monitoring the release of interleukin (IL)-2 by DO.11 using a solid phase enzyme-linked immunosorbent assay (ELISA) (Pharmingen). There was relatively little presentation in cells fixed directly after antigen uptake, although at the highest concentration of antigen, presentation was similar to that shown by A20 B cells. During the next 24–48 h, the presentation capacity of the DCs increased dramatically with maturation, but A20 presentation did not change significantly.

class II–peptide complexes formed in early cells survive to reach the surface, then ‘productive’ peptide loading may occur only in cells that have received a signal to initiate maturation and thus to deliver new class II molecules to the surface. These signals may be provided by cytokines or bacterial products upon the advent of infection, so antigen-presenting capacity may be coupled to inflammatory stimuli and thus the presence of foreign antigens in need of presentation. Without an inflammatory signal, DCs in the periphery should encounter only self antigens, the presentation of which by mature DCs in the periphery might break T-cell tolerance. Such a situation may be a cause of chronic inflammatory lesions²⁰.

The ability of stimulated DCs to decrease their rate of class II degradation and increase the expression of surface class II molecules is a fundamental feature of DC maturation and is also exhibited by a very different population of DCs, those derived from human peripheral blood monocytes²¹. Although ‘immature’ human monocyte-derived DCs appear to be capable of antigen presentation²¹, they may represent a later developmental stage than our bone-marrow-derived early DCs, or they may mature during T-cell assay. But both DC populations effectively retain the memory of antigens internalized earlier in development. □

Methods

Cell culture. Male BDF1 7–8-week-old mice were purchased from Charles River Laboratories (Cambridge, Massachusetts) and bone-marrow-derived DCs cultured as described⁸. Rabbit complement and monoclonal antibodies TIB120, GK1.5, TIB 211 and B220 were obtained from Pharmingen or as hybridomas (from ATCC). Recombinant murine GM-CSF was produced as a culture supernatant from J558L cells transfected with murine GM-CSF cDNA (gift from D. Gray). For some experiments, purified yeast GM-CSF (Kirin Brewing, Japan) was used, with equivalent results. Cells were washed every 2 days to remove non-adherent granulocytes and at day 6, adherent DCs, which were predominantly of the ‘early’ phenotype, were dislodged and replated in 10-cm dishes with fresh medium to generate a culture enriched in intermediate, and then in late cells by culturing for 1 and 3 days, respectively.

Cell clusters were purified by unit-gravity sedimentation on 6 ml 50% FCS columns⁸, followed by depletion for GR1-positive and/or surface class-II-positive cells by sterile cell-sorting using a Becton–Dickinson FACStar. Epidermal sheets from mouse ears were prepared as described^{15,16} and kept in RPMI 1640 supplemented with 5% FCS. Langerhans cells (LCs) were isolated by treatment of the epidermis with collagenase¹⁶ and cultivated in RPMI 1640 supplemented with 5% FCS and 1% GM-CSF.

Antibodies and immunocytochemistry. Immunofluorescence patterns were visualized by confocal microscopy as described¹². Murine I-A was detected using Rivoli affinity-purified rabbit polyclonal antibody directed against the conserved cytoplasmic tail peptide (KGPRGPPAGLLQ) of all class II I–A β -chains; H2-M was detected with ‘Ulm’ affinity-purified rabbit polyclonal antibody directed against the cytoplasmic tail peptide (KYTPLSGSTYPEGRH) of the H2-M β -chain; murine IgG-B was detected using a rat anti-mouse monoclonal antibody, GL2A7 (ref. 12). The anti-class II antibodies Y3P and M5514 antibodies are described elsewhere²² and anti LFA1/CD18 (Rb320) was a gift from P. Blier, Boehringer–Ingelheim). Anti-GR1 and Ser-4 antibodies used for immunofluorescence and FACS were obtained from Pharmingen.

Free-flow electrophoresis. 4×10^7 DCs were grown up and homogenized in 3 ml TEA buffer (10 mM triethanolamine, 1 mM EDTA, 10 mM acetic acid) with 250 mM sucrose, pH 7.4, by 10 passes in a ball-bearing homogenizer (26- μ m gap). Post-nuclear supernatants were fractionated by flotation (90 min at 37,000 r.p.m. in a Beckman SW41-Ti rotor) in a discontinuous sucrose-density gradient (1.4, 1.25, 0.25 M sucrose in TEA buffer). Low-density membranes enriched in endosomes (1.25–0.25 M interface) were collected, adjusted to 0.24 M in sucrose and briefly trypsinized (3 μ g trypsin per mg protein at 37 °C for 10 min). The reaction was stopped with an excess of soya bean trypsin inhibitor. The sample was injected into a Dr Weber GmbH Octopus PSZ free-flow electrophoresis chamber at a rate of 100 ml min⁻¹, and run at a flow rate of 250 ml h⁻¹ at 110 mA. The marker enzymes, the protein assay and details of the electrophoresis have been described^{13,23}. The distribution of MHC class II and LFA-1 was determined by western blotting of membranes collected by centrifugation (30 min at 14,000 r.p.m.).

Immunoprecipitation and cell-surface biotinylation. 3×10^7 DCs were pulse-labelled with 10 mCi in 5 ml of [³⁵S]methionine Translabel (ICN Biochemicals) in labelling medium for 20 min and chased for various times at 37 °C in RPMI/10% FCS with a 15-fold excess of cold methionine and cysteine as described^{22,24}. Radiolabelled cell pellets were lysed for 10 min on ice in 10 mM Tris, 150 mM NaCl (pH 7.4), containing 1% TX-100 (Sigma), 0.5 mM PMSF and protease-inhibitor cocktails²⁴. Lysates were pre-cleared with protein A–Sepharose (Pharmacia) at 4 °C. For surface biotinylation, intact cells were resuspended in 1 ml PBS (pH 8) and incubated with 2 mg ml⁻¹ of N-hydroxy-succinamide-S-S-biotin (HHS-SS-biotin; Pierce) for 3 min on ice as described²⁴. Lysates were immunoprecipitated overnight with Y3P anti-class II rat mAb and protein A–Sepharose. Biotinylated samples were eluted from the beads in 100 μ l PBS containing 2% SDS. 20 μ l were transferred to SDS–PAGE sample buffer (total) and the remaining 80 μ l diluted into 1 ml PBS containing 1% Empigen. Biotinylated proteins were recovered from the 80- μ l aliquot by adsorption to streptavidin–agarose beads (Pierce) for 2 h at 25 °C, which were then washed 3 times in precipitation buffer and transferred to SDS–PAGE sample buffer (surface). Immunoprecipitates were analysed on 12% gels and quantified with a Molecular Dynamics phosphorimager or by image digitization using a Visage 2000 image processor (BioImage). The efficiency of class II appearance at the plasma membrane was determined by calculating²⁴ the fraction of biotinylated class II (recovered by streptavidin–agarose) as a percentage of the total labelled class II (immunoprecipitated by Y3P from the initial cell lysate).

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Distinct actions of *cis* and *trans* ATP within the double ring of the chaperonin GroEL

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The chaperonin GroEL is a double-ring structure with a central cavity in each ring that provides an environment for the efficient folding of proteins^{1–3} when capped by the co-chaperone GroES in the presence of adenine nucleotides^{4–8}. Productive folding of the substrate rhodanese has been observed in *cis* ternary complexes, where GroES and polypeptide are bound to the same ring, formed with either ATP, ADP or non-hydrolysable ATP analogues^{2,9}, suggesting that the specific requirement for ATP is confined to an action in the *trans* ring that evicts GroES and polypeptide from the *cis* side⁹. We show here, however, that for the folding of malate dehydrogenase and Rubisco there is also an absolute requirement for ATP in the *cis* ring, as ADP and AMP-PNP are unable to

promote folding. We investigated the specific roles of binding and hydrolysis of ATP in the *cis* and *trans* rings using mutant forms of GroEL that bind ATP but are defective in its hydrolysis. Binding of ATP and GroES in *cis* initiated productive folding inside a highly stable GroEL–ATP–GroES complex. To discharge GroES and polypeptide, ATP hydrolysis in the *cis* ring was required to form a GroEL–ADP–GroES complex with decreased stability, priming the *cis* complex for release by ATP binding (without hydrolysis) in the *trans* ring. These observations offer an explanation of why GroEL functions as a double-ring complex.

The monomeric protein rhodanese has recently been shown to reach native form inside *cis* ternary GroEL–GroES complexes that were formed in ATP, AMP-PNP or ADP, albeit at different rates (ATP > AMP-PNP > ADP)^{2,9}. The single-ring GroEL mutant SR1 has been used to produce obligate and stable *cis* complexes for study^{1,2,9}. Because it has no second ring, the SR1 mutant does not receive the signal from *trans*-sided ATP that normally evicts GroES and substrate⁶. After GroES and any of the three nucleotides were added to rhodanese–SR1 binary complexes, productive folding was shown to occur in the *cis* cavity. We observed that rhodanese that was refolded inside SR1–GroES formed in the presence of ATP could be released efficiently to the medium by brief incubation at 4 °C, a treatment previously shown to lead to rapid dissociation of GroES from an ADP complex with GroEL⁶. Similarly, folding of ornithine transcarbamylase (OTC) from binary complexes with SR1 occurred in the presence of GroES, with almost identical kinetics with either ATP or ADP, when GroES was released by incubation at 4 °C, thereby allowing OTC trimerization (data not shown). These data suggest that the previous observation of OTC folding in the presence of ATP in a single turnover from a *cis* ternary complex¹ resulted from ADP-driven folding during preparation of the complex, with subsequent release when ATP was added. In experiments using SR1 and the green fluorescent protein (GFP), which also refolds inside *cis* complexes formed with any of the three nucleotides, brief treatment at 4 °C also leads to efficient release of GFP (Fig. 1a).

When the same tests were performed with two stringent substrate proteins, Rubisco from *Rhodospirillum rubrum*^{10,11} and mitochondrial malate dehydrogenase (MDH) from pig heart^{12–14}, we observed that only ATP could promote reactivation from wild-type or SR1 *cis* ternary complexes (Fig. 1b, c). Release of GroES from SR1 by treatment at 4 °C was essential for production of enzymatic activity, because both Rubisco and MDH are homodimers, requiring assembly of the refolded monomeric subunits to reach active form. Direct incubation at 4 °C of metastable intermediates of Rubisco that were produced after dilution from denaturant (into the same chloride-free buffer used in all of the Rubisco studies)⁶ did not result in enzymatic activity (data not shown). Remarkably, the kinetics of reactivation by the SR1–GroES ternary complexes in ATP were similar to, if not faster than, those achieved in similar reactions with wild-type GroEL, indicating that a stable folding-active state is produced at SR1. Addition of 'trap' molecules (such as 337/349)¹⁵, which are able to bind but not release non-native substrate proteins, at the time of cold release of GroES had no effect on the kinetics of reactivation (kinetics not shown, but see Fig. 1f, traces 1, 4). These data indicate that, as with rhodanese, folding of both Rubisco and MDH proceeded to a committed state in the ATP *cis* ternary complexes; that is, the substrates reached conformations no longer recognizable by chaperonin.

We wished to follow directly the folding of substrate within the various *cis* complexes, thereby obviating any requirement for the release of GroES and peptide to assay enzymatic activity. We therefore examined by stopped-flow changes in the fluorescence total intensity and anisotropy of tryptophan residues of Rubisco in complexes formed following the addition of GroES and nucleotides to Rubisco–chaperonin binary complexes (Fig. 1d, e). This takes advantage of the absence of tryptophan from both GroEL and

GroES, and so allows us to monitor the conformational changes occurring in the substrate during the chaperonin reaction^{16–18}. Addition of ATP and GroES to complexes of either SR1–Rubisco or wild-type GroEL–Rubisco resulted in an initial rapid drop in total fluorescence intensity and anisotropy ($t_{1/2} \sim 1$ s), followed by a

steady rise in both intensity and anisotropy ($t_{1/2} \sim 3.5$ min) (Fig. 1d, e; only anisotropy data are shown). The rate of the rising phase corresponded within a factor of two with the rate of recovery of Rubisco enzymatic activity from both SR1 and wild-type GroEL (Fig. 1b). The early decrease in anisotropy probably indicates the

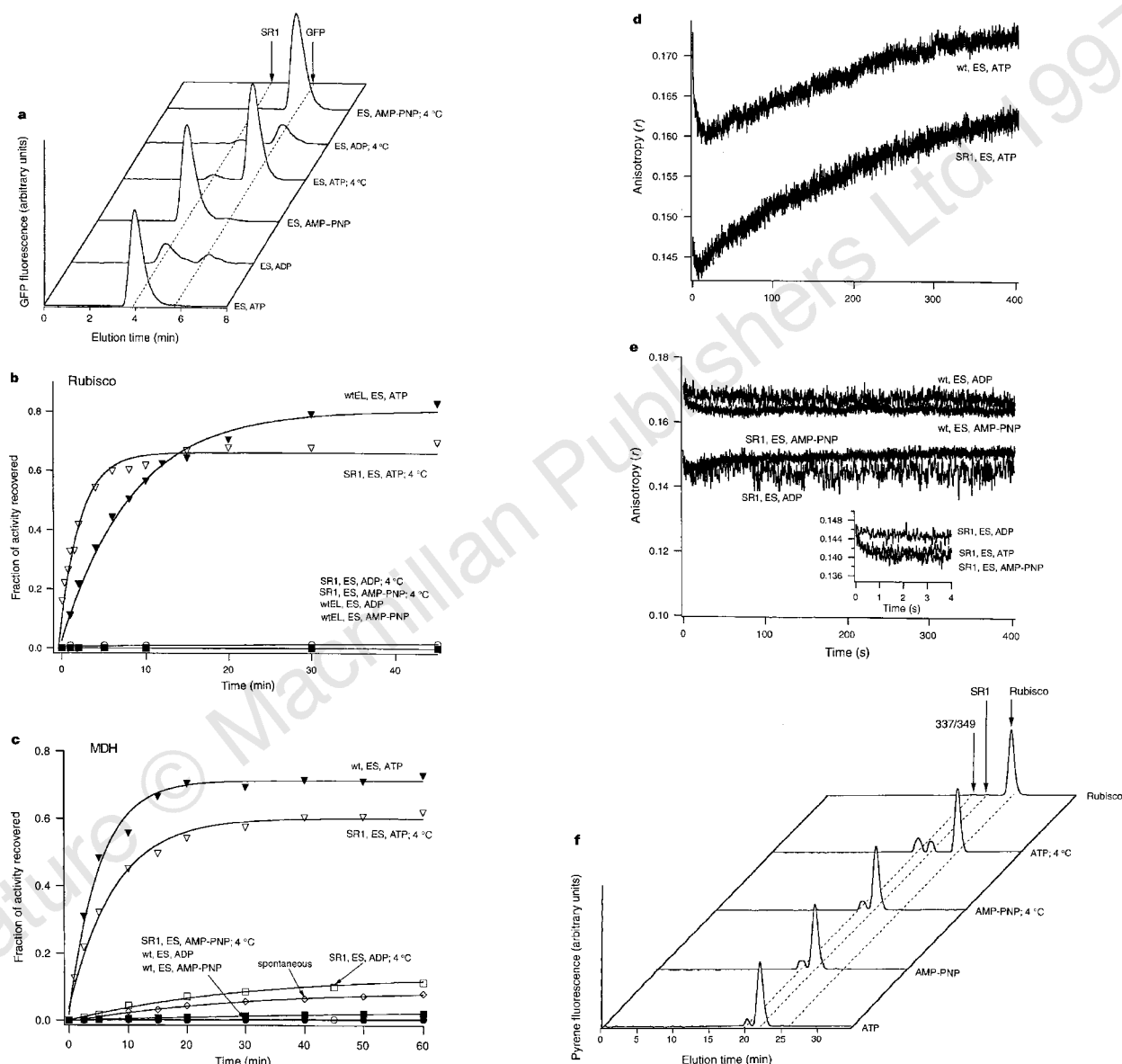


Figure 1 ATP is required in *cis* for the GroEL–GroES-mediated folding of two stringent substrates, Rubisco and MDH. **a**, Folding of the non-stringent substrate GFP inside the complex formed between the single-ring mutant, SR1, and GroES, and release of folded GFP by 4 °C treatment. Binary complexes between acid-denatured GFP and SR1 were prepared and mixed with GroES and either ATP, ADP or AMP-PNP. Samples of the complexes, with folded, trapped GFP inside, were incubated for 10 min at either 23 °C (unlabelled) or 4 °C, then subjected to gel filtration with on-line fluorescence detection. **b**, Refolding of Rubisco by wild-type GroEL and SR1. Binary complexes were formed between acid-denatured Rubisco, and either wild-type GroEL (wtEL) or SR1, and GroES (ES) and nucleotide were added. SR1 reactions were incubated at 4 °C for 15 min before enzyme assay to release bound GroES and free folded Rubisco monomers. SR1 reactions incubated at 23 °C demonstrated no Rubisco activity. Incubation of acid-denatured Rubisco for various times at 4 °C without chaperonin resulted in a recovery of only 1–2% activity. Total recovery is based on the activity of 100 nM native Rubisco (monomer). **c**, Refolding of mitochondrial MDH by wild-type GroEL and SR1. Refolding of MDH in binary wild-type GroEL–MDH or SR1–MDH complexes was initiated by the addition of GroES and nucleotide. The spontaneous recovery of refolded MDH in the absence of chaperonins was approximately 10%. Assaying SR1 reactions for enzymatic activity without prior incubation at 4 °C resulted in <4% recovery of activity. **d, e**, Folding of Rubisco with wild-type GroEL and SR1 monitored by changes in intrinsic tryptophan fluorescence anisotropy. Binary complexes were formed between acid-denatured Rubisco and either wild-type GroEL or SR1 and rapidly mixed (1 : 1) in a stopped-flow apparatus with solutions containing GroES and either ATP (**d**), ADP or AMP-PNP (**e**). The anisotropy of the Rubisco intrinsic tryptophan fluorescence was monitored as a function of time after mixing. Traces represent summations of 25 individual experiments (**d**) or 10–15 experiments (**e**). Inset, early time measurements with SR1; each trace is the sum of 40–50 experiments. **f**, Failure of Rubisco to fold in SR1–GroES–AMP–PNP complexes correlates with inability of the polypeptide to dissociate from SR1. Binary complexes were formed between acid-denatured RUBPyr and SR1 and mixed with GroES and either ATP or AMP-PNP. After 1 h at 23 °C, 337/349 GroEL trap tetradecamer was added, and the mixture incubated at either 23 °C or 4 °C for an additional 10 min before gel filtration. Based on proteolytic protection experiments (not shown), GroES binds as stably to the SR1–Rubisco complex in AMP-PNP as in ATP.

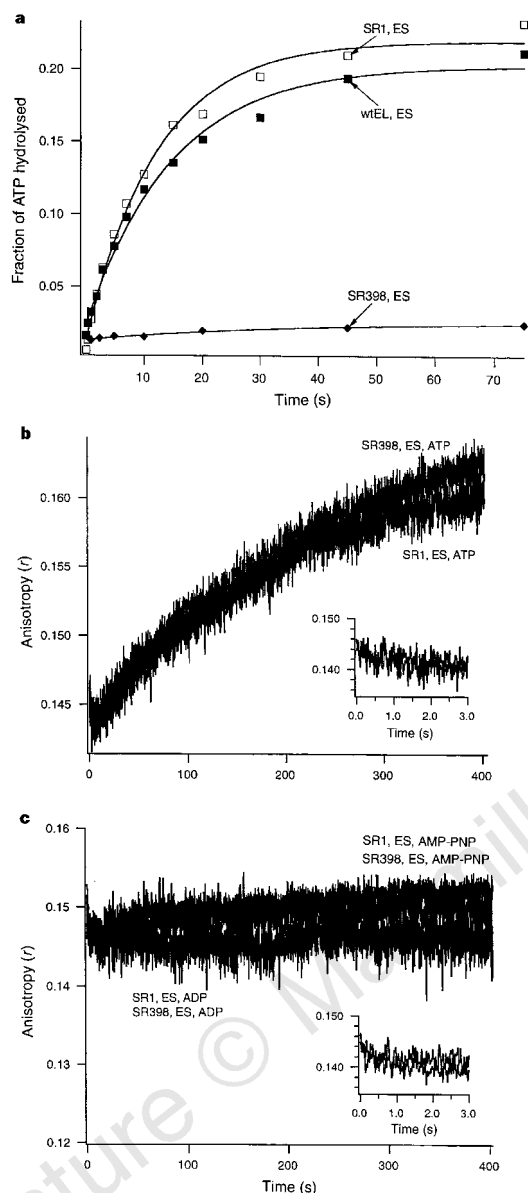


Figure 2 Binding of ATP and GroES to the *cis* ring of GroEL is sufficient to trigger the release and folding of Rubisco. **a**, ATPase activity of wild-type GroEL (wtEL), SR1 and SR398 in the presence of GroES (ES) was measured by rapid mixing/quenching in a quench-flow apparatus. Experiments with binary complexes between each of these chaperonins and Rubisco showed no significant difference in hydrolytic activity. **b**, **c**, The SR398 ring is competent to fold Rubisco, despite absence of significant ATP hydrolytic activity. Binary complexes were formed between acid-denatured Rubisco and SR398 and rapidly mixed (1:1) in a stopped-flow apparatus with solutions containing GroES and either ATP (**b**), ADP (**c**) or AMP-PNP (**c**). Data for SR1 from Fig. 1 are shown again for comparison. Traces represent the summation of 25 (**b**) or 10–15 (**c**) experiments. Inset, early changes in anisotropy of the SR1 and SR398 complexes with ATP (**b**) and AMP-PNP (**c**). **d**, High affinity of GroES for SR398 in ATP, and recovery of refolded GFP and Rubisco from SR398 ternary complexes by proteolytic treatment. GFP–SR398 binary complexes were mixed with GroES and ATP and the mixture gel filtered at 23 °C with monitoring of fluorescence in-line. Exposure of the purified ternary complexes to 4 °C or 250 mM KOAc at 4 °C failed to significantly release the bound substrate. Exposure to 10% methanol or 0.4 M guanidine-HCl (GdHCl) led to only partial release. Treatment with trypsin followed by 4 °C incubation leads to full release of GFP. For recovery of refolded Rubisco from SR398–GroES–ATP ternary complexes, a similar treatment was performed using proteinase K followed by assay for enzymatic activity (inset).

rapid mobilization of reporting regions of the Rubisco polypeptide chain, associated with release of polypeptide from the apical binding sites. The slower increase in intensity and anisotropy, associated with recovery of activity, probably reflects the burial and packing of the tryptophan side chains into the core of the refolding Rubisco monomer. In contrast, these slow rising changes were not observed when ADP or AMP-PNP was substituted for ATP, correlating with the failure of these nucleotides to support production of the native state (Fig. 1e). However, AMP-PNP, but not ADP, induced the same initial rapid drop of both intensity and anisotropy that was observed with ATP (Fig. 1e, inset), suggesting that binding of GroES with AMP-PNP produces some of the same early local motion in Rubisco. However, given that no further anisotropy changes occurred (although there was a slow drop in intensity), it seems that the polypeptide is either not fully released or is recaptured by the apical peptide-binding surface¹⁹.

The ongoing association of Rubisco with chaperonin in AMP-PNP was observed directly in an experiment with SR1 and RUBpyr, which is a pyrene-labelled derivative of Rubisco. RUBpyr bears a single pyrene that does not interfere with catalytic function and allows GroEL-mediated refolding to native form, which occurs as completely as with unmodified Rubisco but at a slower rate. As

shown in the gel filtration profiles (Fig. 1f), RUBpyr was efficiently refolded when GroES and ATP were added to SR1–RUBpyr binary complex, because, after release with treatment at 4 °C, RUBpyr migrated to the position of the Rubisco homodimer. In contrast, however, when GroES and AMP-PNP were added to the binary complex, RUBpyr comigrated with SR1 after treatment at 4 °C, indicating that the non-native form remained bound to SR1, unable to be captured by added 337/349 trap (Fig. 1f). Thus both anisotropy and gel-filtration studies indicate that the formation of the *cis* complex in ATP releases Rubisco into the central channel, initiating folding, whereas formation of *cis* complex in AMP-PNP (or ADP) does not fully release the polypeptide. AMP-PNP does not seem to produce the same stereochemical changes in GroEL–GroES complexes as ATP²⁰, reflecting the fact that AMP-PNP is not a perfect ATP homologue.

These studies demonstrated that productive conformational changes of substrate protein were triggered when ATP and GroES were added, but it was not clear whether the immediate trigger to folding was initial ATP–GroES binding or subsequent ATP hydrolysis. To resolve this question, we examined a mutant in which Asp 398 in the intermediate domain was affected, a residue shown in the GroEL–GroES–ADP crystal structure to have moved from a remote location in the unliganded structure into the equatorial

nucleotide-binding site to a position just distal to the β -phosphate of ADP, and which contributes to the ligation of Mg^{2+} (ref. 19). Consistent with the structural analysis, the D398A mutant was able to bind ATP with normal affinity, but was defective in ATP turnover,

with $\sim 2\%$ wild-type activity (data not shown). As expected, the D398A tetradecamer bound non-native rhodanese and, in the presence of GroES and ATP, formed a complex that could mediate production of native rhodanese but could not release the protein (data not shown). Similarly, when this mutation was introduced into SR1, the derived SR398 complex bound ATP and GroES in the absence of significant hydrolysis (Fig. 2a) and refolded rhodanese in the ternary complex without releasing the native protein. Fluorescence-dynamics studies were also performed with SR398–Rubisco complexes. When GroES and ATP were mixed rapidly, total intensity and anisotropy changes were observed that were virtually identical to those seen with the hydrolysis-proficient SR1 complex (Fig. 2b, c). Once again, when AMP-PNP or ADP were used instead of ATP, the rising (productive) phase of anisotropy/intensity was absent, although AMP-PNP again promoted an early drop of anisotropy in SR398-bound Rubisco. We conclude that ATP–GroES binding alone is sufficient to trigger Rubisco release and productive folding in *cis* ternary complexes.

To determine whether Rubisco subunits could reach native form inside the SR398–GroES ternary complex, we sought to measure enzyme activity after incubation at 4°C , as with SR1. Surprisingly, no activity was detected, owing to the failure of GroES to release from the SR398 complex. This failure was demonstrated by gel filtration of ^{35}S -GroES–SR398–Rubisco ternary complexes that had been exposed to incubation at 4°C , where ^{35}S -GroES migrated with the complex (data not shown). This was further supported by gel filtration of SR398–GroES–GFP complexes in which, after incubation at 4°C , the fluorescent refolded GFP remained associated with SR398 (Fig. 2d). This latter analysis indicated that even transient release of GroES, which would allow escape of GFP, did not occur. Remarkably, much more severe treatments, including incubation in 0.4 M guanidine HCl, also failed to release GFP from SR398–GroES–ATP (Fig. 2d). We finally resorted to partial proteolytic treatment with trypsin, which released intact, fluorescent GFP from the chaperonin complex (Fig. 2d). When a similar proteolytic step was performed with Rubisco bound in SR398–GroES–ATP, we observed consistent recovery of significant activity (Fig. 2d; 4–6% in each of three independent experiments). Significantly, the same low level of recovery was observed after proteolysis of the productive SR1 ternary complex (from which $>60\%$ activity was recovered if 4°C release was used instead, as in Fig. 1b). The low recovery in both cases presumably results from unintended proteolytic digestion of refolded substrate protein. These findings support the indications from fluorescence studies that binding of ATP and GroES is sufficient to trigger folding of at least a fraction of Rubisco molecules to native form in the SR398 complex.

The high affinity of GroEL for GroES in ATP revealed by the study of hydrolysis-defective D398A mutants suggested that, under normal conditions, the *cis* ring must undergo hydrolysis of bound ATP before ATP in the *trans* ring can trigger GroES release. The ADP-bound state presumably comprises a lower-affinity GroEL–GroES interaction, or is more responsive to a signal for release of GroES produced by the *trans* ring. To test this, we produced a mixed-ring GroEL assembly²¹, composed of a D398A ring, which was able to bind polypeptide and GroES but unable to hydrolyse ATP, and a 203/337/349 (3-7-9) ring, which was unable to bind substrate or GroES but able to provide normal ATP function in *trans* to the D398A ring (see Fig. 3a). The mixed-ring complex, MR2, was tested for ability to stably bind ^{35}S -labelled GroES in ATP or ADP. In the presence of ATP, MR2 stably bound GroES, failing to release it even though there was hydrolysis in the 3-7-9 ring (at a rate $\sim 21\%$ of wild-type, unaffected by GroES). In particular, when SR398 was added as a GroES 'trap' to a mixture containing MR2–GroES and ATP, no transfer of radiolabelled GroES from MR2 to SR398 was observed by gel-filtration analysis (Fig. 3b). Thus the high affinity for GroES of the D398A ring of MR2 in ATP seems to recapitulate that observed with SR398 in ATP. GroES was also stably bound to

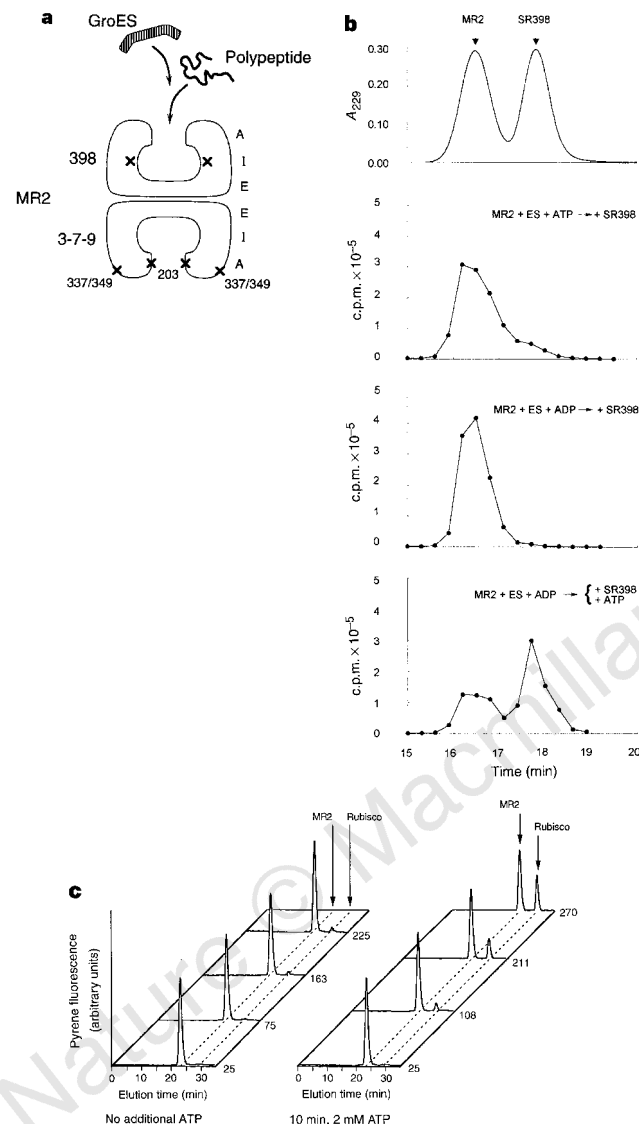


Figure 3 ATP hydrolysis in the *cis* ring 'primes' release of GroES and peptide ligands by *trans*-sided ATP. **a**, Schematic representation of the mixed-ring GroEL complex, MR2. The ring containing D398A mutant subunits can bind polypeptide and GroES but is defective in ATP hydrolysis; the 3-7-9 ring cannot bind polypeptide or GroES as a result of apical substitutions in its subunits, but is proficient in ATP hydrolysis²¹. Apical (A), intermediate (I) and equatorial (E) domains of the GroEL subunits are shown; X, mutations. **b**, ATP triggers release of GroES bound to MR2 in ADP but not in ATP. Gel filtration analyses are shown of chaperonin mixtures in which the single-ring GroES 'trap' SR398, was added to capture GroES released from binary complexes formed between MR2 and ^{35}S -radiolabelled GroES. Top, A_{229} profile of MR2 and SR398; middle panels, MR2–GroES complexes formed in either ATP or ADP do not release GroES. Bottom, addition of ATP triggers release of GroES from MR2–GroES binary complex formed in ADP. **c**, Rubisco refolded in MR2–GroES–ATP complexes can be productively released by addition of ATP after prolonged incubation of the purified ternary complexes. RUBpyr–MR2 binary complexes were incubated with GroES in the presence of ATP and the ternary complexes purified by gel filtration in the absence of nucleotide. Portions of ternary complex were exposed to ATP at the indicated time at 23°C after initial mixing, and mixtures were fractionated by gel filtration with in-line fluorescence detection. Migration positions of MR2 and native homodimeric Rubisco are shown.

MR2 in ADP, although if ADP was absent from the gel filtration running buffer, the GroES was released (data not shown). This would seem to be consistent with an apparently lower affinity interaction of GroES with the D398A ring in ADP (compared with no release from the ATP complex when it was gel filtered in the absence of nucleotide). More importantly, however, when the MR2–GroES complex formed in ADP was exposed to ATP, GroES was released efficiently, and was detected through its capture by co-incubated SR398 (Fig. 3b). This suggests that the pre-bound ADP *cis* complex emulates the post-hydrolysis ADP *cis* complex in being similarly coupled to the action of *trans*-sided ATP, allowing GroES to be released.

The consequences of *cis* ATP hydrolysis for release of folded polypeptide were examined more directly by forming RUBpyr–MR2–GroES complexes in ATP, purifying them by gel filtration, and incubating them in the absence of nucleotide for prolonged periods, during which the bound ATP slowly hydrolysed in the D398A ring. When such complexes were then exposed to ATP and the reaction mixtures gel filtered, there was a progressive increase with incubation time in recovery of fluorescence at the position of native homodimeric Rubisco, reflecting the gradual priming of the *cis* ring for *trans*-ATP-driven release of the folded RUBpyr (Fig. 3c).

Once we had observed the specific requirement for binding of ATP in the *cis* ring to promote folding, with AMP-PNP unable to substitute, we then investigated whether a similar requirement might be operative in the *trans* ring with respect to driving GroES/polypeptide release from the *cis* side. Previous studies have observed that AMP-PNP and ATP- γ S supplied in *trans* are unable to support the release of GroES from *cis*, but have been interpreted to indicate that hydrolysis is required in *trans* for eviction from *cis*⁶. To address this question, the hydrolysis-defective D398A tetradecamer was used to form folding-active *cis* ternary complexes in ATP which were allowed to slowly hydrolyse bound *cis* ATP over 2 h to produce a 'primed' *cis*-ADP state (as in Fig. 3c). The complexes were then incubated briefly with ADP, AMP-PNP or ATP. When GFP was examined as a substrate, gel-filtration studies showed that the folded protein remained stably associated in D398A ternary com-

plexes (purified away from the nucleotide) over the 2-h time period. GFP was not released upon subsequent addition of ADP or AMP-PNP (Fig. 4). However, when ATP was added, gel filtration after 2 min showed that >60% of the GFP was released (Fig. 4), which corresponds to the efficiency of GFP release observed when ATP was added to a wild-type *cis*-ADP–GFP complex (Fig. 4). MDH was also used as a substrate in a similar experiment (not shown), in which limiting amounts (just sufficient for formation of *cis* complex) of ATP were supplied, followed by incubation for 2 h. Once again, brief exposure to ATP but not ADP or AMP-PNP produced recovery of enzymatic activity, the apparent result of release of refolded monomer from the *cis* ternary ADP complex only by ATP. These studies with D398A indicate that binding of ATP in *trans*, independent of hydrolysis, is sufficient to promote the release of GroES and polypeptide from *cis*-ADP complexes.

In summary, our results indicate that GroEL-mediated folding of stringent substrates like Rubisco and MDH requires action of ATP in both the *cis* and *trans* rings (Fig. 5). In the *cis* ring, binding of ATP and GroES triggers folding, potentially beginning within 1 s, a finding that is in accord with the early and rapid drop of anisotropy reported here and elsewhere². ATP, specifically, promotes the complete release of stringent substrates like Rubisco into the central channel, which enlarges at least twofold in volume in association with GroES binding and changes in surface character from hydrophobic to hydrophilic¹⁹ (Fig. 1d, f). Hydrolysis of ATP in the *cis* ring, occurring with a $t_{1/2}$ of 6–8 s (see Fig. 2a and ref. 7), is then necessary to relax the high-affinity interaction of GroES with ATP-bound GroEL, priming the ring for the release of GroES, which is accomplished by subsequent binding of ATP in the *trans* ring.

Methods

Proteins. Tetradecameric GroEL with the D398A substitution (D398A) was produced by polymerase chain reaction (PCR)-mediated site-directed mutagenesis of a plasmid encoding wild-type GroEL²¹. A single-ring mutant containing the D398A substitution (SR398) was produced by exchanging a

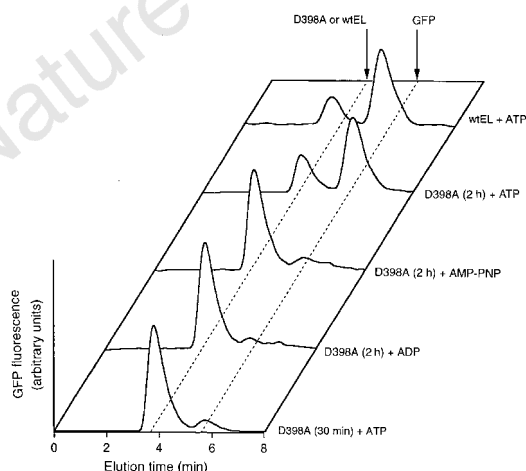


Figure 4 Release of GFP refolded in tetradecameric D398A *cis* ternary complexes by subsequent addition in *trans* of ATP but not ADP or AMP-PNP. GFP–D398A binary complexes were incubated with GroES and ATP, treated briefly with proteinase K to remove any GFP bound in *trans*, then purified by gel filtration in the absence of nucleotide. The complexes were then incubated for 30 min or 2 h, then exposed to ATP, ADP or AMP-PNP for 2 min and rapidly gel filtered with in-line fluorescence detection. By 2 h the amount of GFP released by ATP from D398A tetradecamer is similar to that obtained from GFP–wtGroEL–GroES–ADP *cis* complexes exposed to ATP for 1 min. ATP triggered release from D398A, but ADP and AMP-PNP had no effect.

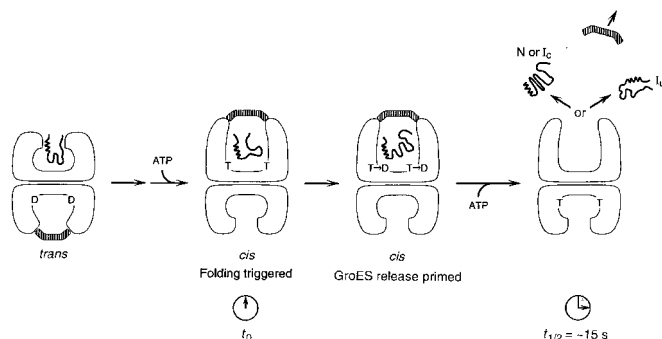


Figure 5 Schematic representation of the action of ATP in *cis* and *trans* rings during GroEL–GroES-mediated protein folding. Polypeptide is initially bound in the *trans* ring of a GroEL–GroES binary complex, and a step of ATP-triggered GroES release and rebinding or of binding of a second GroES to the polypeptide-bound ring occurs, forming a *cis* complex in which polypeptide is sequestered in the GroEL central channel underneath GroES^{23,24}. The act of formation of the *cis* complex, through binding of ATP and GroES to the ring with bound polypeptide, triggers rapid release of polypeptide from the apical binding sites into the central channel, and folding begins (t_0) (see Figs 1, 2). Hydrolysis of ATP in the *cis* ring weakens the high-affinity interaction between GroES and (*cis*)ATP-bound GroEL (see Fig. 3), and binding of ATP in *trans* subsequently produces release of GroES and polypeptide from the *cis* ring ($t_{1/2}$ ~ 15 s) (see Fig. 4). D, ADP; T, ATP; T $\rightarrow$ D, hydrolysis of ATP; N, native; I_c , non-native conformation committed to completing folding to native form (no longer recognizable by chaperonin); I_{nc} , non-native form that must be rebound by chaperonin or by a different chaperone to reach native form.

restriction fragment with coding sequences bearing the D398A mutation for the wild-type segment with that in an expression plasmid encoding SR1. GroEL, SR1 and the derivatives, as well as GroES, were expressed in *Escherichia coli* and purified as described¹. Metabolically labelled [³⁵S]GroES was produced as described¹. Pig heart mitochondrial malate dehydrogenase (Sigma) was purified by gel filtration chromatography. Soluble, fluorescent GFP and Rubisco from *Rhodospirillum rubrum* were expressed at 20 °C from T7 promoters in BL21 transformants in the absence of induction and were purified by ion-exchange chromatography.

GFP refolding. All GFP folding experiments were conducted in buffer A (50 mM HEPES, pH 7.6, 5 mM KOAc, 10 mM Mg(OAc)₂, 1 mM DTT) at 23 °C. GFP (~530 μM) was acid-denatured by a 5-fold dilution with 25 mM glycine-phosphate buffer (pH 2.0). After 5 min at 23 °C, binary complexes were generated by diluting the denatured GFP 11-fold (9 μM final concentration) into 27 μM SR1 (Fig. 1a) or SR398 (Fig. 2d) in buffer A and incubating for 10 min at 23 °C. For SR1 experiments (Fig. 1a), GroES was added to 50 μM and either ATP to 1.5 mM or AMP-PNP to 5 mM, or GroES was added to 165 μM and ADP to 1.5 mM. Folding mixtures were incubated at 23 °C for 45 min. One portion was placed at 4 °C for 10 min, while another was maintained at 23 °C. All samples were then chromatographed over three Tosohaas TSK-gel guard columns connected in series. In-line fluorescence detection of renatured GFP used excitation at 339 nm and an emission cut-off filter centred at 495 nm. For SR398 experiments (Fig. 2d), GroES was added to 66 μM and ATP to 2 mM, and the mixture was incubated at 23 °C for 15 min, then gel filtered on the guard columns. The purified ternary complex was either reinjected or first treated for 15 min with the indicated conditions. For protease treatment, one portion was supplemented with trypsin to 15 μg ml⁻¹ and incubated at 23 °C for 10 min. Soybean trypsin inhibitor was then added to 125 μg ml⁻¹, the sample incubated at 4 °C for 10 min, and then injected. For D398A (tetradecamer) studies (Fig. 4), ternary complex was formed as above. After 15 min at 23 °C, proteinase K was added (1 μg ml⁻¹ final concentration) to remove GFP bound in *trans*. After 5 min, PMSF was added to 1 mM and the complex was purified by gel filtration on the tandem guard columns in buffer A. The purified complex was incubated in the dark at 23 °C for 30 min or 2 h. Samples were then supplemented with either ATP (2 mM), ADP (5 mM) or AMP-PNP (5 mM), incubated for 2 min, then reinjected to the guard columns and analysed by on-line fluorescence detection. Wild-type GroEL–GroES–GFP complex was prepared similarly, except that ADP (1 mM) and GroES 150 μM were used. Despite the low yield of this assembly, the ADP-ternary complex was stable until challenged with ATP, as determined by rechromatography.

Rubisco refolding. Rubisco was denatured by a 1:55 dilution into 25 mM glycine-phosphate buffer, pH 2.0. Immediately after a 3–5 min incubation at 23 °C, binary complexes were formed by mixing the unfolded Rubisco with chaperonin in a total volume of 1.5 ml buffer A. The final concentrations were 130 nM wild-type GroEL, 300 nM SR1 or SR398, and 100 nM Rubisco (monomer). Folding was initiated by addition of a two-fold molar excess of GroES (over chaperonin) and either ATP (1 mM), ADP (5 mM) or AMP-PNP (5 mM) (final concentrations). For wild-type reactions, at the times indicated, ATP was removed by adding hexokinase and glucose (final concentrations, 0.08 U μl⁻¹ hexokinase and 10 mM glucose). For SR1 reactions, ATP was removed 30 s after its addition by adding hexokinase and glucose. SR1 reactions were incubated at 4 °C for 15 min before enzyme assay to release bound GroES and free folded Rubisco monomers. SR1 samples were briefly allowed to re-equilibrate to 23 °C before enzyme assay. For proteolytic release of Rubisco from SR398–GroES and SR1–GroES ternary complexes (Fig. 2d), after 7 min of refolding at 23 °C (see Fig. 1b) the mixture was supplemented to 3 mM Ca(OAc)₂ and proteinase K was added to 125 μg ml⁻¹. After incubation at 23 °C for 6 min, PMSF was added to 3 mM and the mixture assayed after a further 10 min at 23 °C for Rubisco activity. Rubisco enzyme activity was determined by incorporation of [¹⁴C]CO₂ into acid-precipitable products¹⁰.

MDH refolding. All MDH refolding studies were performed in 50 mM Tris-HCl, pH 7.4, 50 mM KCl, 10 mM MgCl₂, 10 mM DTT (buffer B). MDH was denatured for 1 h in 5 M urea in buffer B at 30 °C. Binary complexes were formed between wild-type GroEL or SR1 and MDH by rapidly diluting the denatured MDH 250-fold using a solution of 1.2 μM wild-type GroEL or 5 μM

SR1 in buffer B such that the final concentration of MDH was 1 μM with respect to subunits. Refolding was initiated by the addition of a 3-fold molar excess of GroES and either 2 mM ATP, 10 mM ADP or 10 mM AMP-PNP, followed by incubation at 30 °C. At various times a portion was withdrawn and, in the case of wild-type GroEL, assayed immediately for enzymatic activity. In the case of the SR1-mediated reaction, the ATP was removed by hexokinase and glucose as before, then incubated at 4 °C for 10–15 min before assay to release bound GroES and free folded, assembly-competent MDH monomers. For the experiment with D398A, binary complex was formed with 5 μM MDH subunit and 7 μM D398A oligomer (100 μM subunit). GroES and ATP were added to 10 μM and 50 μM final concentrations, respectively. After 2 h at 30 °C, proteinase K was added at 800 ng ml⁻¹, and incubation carried further for 5 min. PMSF was then added to 1 mM. Samples were then supplemented with ATP, AMP-PNP or ADP to 4 mM and after 90 s were assayed for MDH activity. MDH enzyme assay was performed by diluting a portion from the folding assay into 1 mM ketomalonate, 0.2 mM NADH, 10 mM DTT, and 50 mM Tris-HCl, pH 7.4, and following the oxidation of NADH at 340 nm (ref. 14).

Stopped-flow fluorescence anisotropy. The stopped-flow fluorescence anisotropy apparatus used in these studies was constructed by H.S.R. using as a template a design from the laboratory of J. Beechem¹⁶. It consists of a Bio-Logic SFM/4 stopped-flow head coupled to an excitation source and monochromator (Photon Technologies International) through a fibre-optic bundle designed to provide a small slit-shaped (3 × 0.66 mm) excitation beam to the stopped-flow cuvette (FC.15). The two emission channels are collected in T-format through a pair of non-fibre, large core diameter, high numerical aperture (NA 0.59) light guides (Oriel) placed directly against the stopped-flow cuvette to maximize light collection efficiency. Polarization selection of the excitation and emission used film polarizers (Meadowlark Optics) placed over the end of each light guide at the stopped-flow cuvette. The collected fluorescence is spectrally filtered and detected with a pair of Hamamatsu PMTs (R4457P) operated in photon-counting mode. Data collection was as previously described¹⁶. For stopped-flow experiments involving Rubisco, a sample was diluted (1:9) with 25 mM glycine-phosphate buffer, pH 2.0, to produce a 15.6 μM acid-denatured Rubisco stock. Each binary complex was generated by diluting 480 μl of this stock into 5 ml of buffer A containing either wild-type GroEL (2 μM), SR1 (4 μM) or SR398 (4 μM). After 10 min at 23 °C, the binary mixture was loaded into one syringe of the stopped-flow apparatus. The binary mixture was rapidly mixed (1:1) in the stopped-flow (at 23 °C) with a solution containing GroES (6 μM) and either ATP (2 mM), ADP (10 mM) or AMP-PNP (10 mM).

Quenched-flow ATPase assay. ATPase activities of wild-type GroEL, SR1 and SR398 were determined using a quenched-flow rapid mixing apparatus (KinTek) at 20 °C. Equal volumes (15 μl) of chaperonin in buffer A and GroES/[γ-³²P]ATP in buffer A were mixed to give final concentrations of 2.85 μM chaperonin, 6 μM GroES, 100 μM [γ-³²P]ATP (300 μCi μmol⁻¹), then the reaction was quenched with 67 μl 1.5 M formic acid/1.5 M potassium phosphate at the times indicated. Note that the ATP concentration is such that hydrolysis by wild type is not at steady state after one turnover. Portions of each sample were chromatographed on polyethylene imine thin-layer plates, developed with 1 M formic acid and 0.25 M LiCl. Radioactivity in phosphate and ATP regions was quantified by phosphorimager analysis.

Labelling of native Rubisco with pyrene (RUBpyr). Rubisco (0.8 mg ml⁻¹ in 100 mM sodium phosphate, pH 7.6) was reduced with 2 mM TCEP and labelled with a 6–7-fold excess of N-1-pyrene-maleimide, added in two portions. The reaction was quenched by adding glutathione (2 mM), and the labelled Rubisco was separated from unbound dye by gel filtration (PD-10 column, Pharmacia). The labelling ratio was 1:1, pyrene to Rubisco monomer, as determined by absorption spectroscopy, using extinction coefficients of 67,000 M⁻¹ cm⁻¹ at 280 nm for the Rubisco monomer²² and 36,000 M⁻¹ cm⁻¹ at 340 nm for reacted N-1-pyrene-maleimide (Molecular Probes). Labelling of a single cysteine (usually the surface-accessible Cys 58) was indicated by detection of a single major tryptic peptide with strong absorption at 340 nm during separation by C8 reverse-phase chromatography. The specific activity of the labelled enzyme was indistinguishable from that of unlabelled Rubisco, and refolding of acid-denatured RUBpyr was dependent upon GroEL, GroES and

ATP, although renaturation occurred at a rate approximately half that of the unlabelled enzyme.

Refolding and gel filtration with RUBpyr. For RUBpyr refolding, the labelled enzyme was denatured in acid glycine-phosphate buffer as described above. For the experiment shown in Fig. 1, binary complexes were formed by incubating denatured RUBpyr with SR1 in buffer A (final concentrations, 2 μ M RUBpyr and 4 μ M SR1) for at least 10 min at 23 °C. GroES was added to 5 μ M, and folding was initiated by adding either ATP to 1 mM or AMP-PNP to 5 mM. After 1 h at 23 °C, 337/349 GroEL trap was added to 6.5 μ M and incubation continued at either 23 °C or 4 °C for a further 15 min. All samples were then subjected to gel filtration on a TSK G-4000SW_{XL} column equilibrated in buffer A. For the experiment shown in Fig. 3, a 20- μ M solution of unfolded RUBpyr was mixed with buffer A containing MR2 to give final concentrations of 4 μ M RUBpyr and 5 μ M MR2. After 10 min at 23 °C, GroES was added to 10 μ M and ATP to 2 mM, and the mixture incubated at 23 °C for 5 min. Unbound ATP was removed by rapid (4 min) gel filtration over three TSK guard columns connected in series and equilibrated in buffer A. The purified MR2-RUBpyr-GroES complex was incubated at 23 °C for the times indicated, then 200- μ l portions of the complex were either directly injected onto the G-4000 gel filtration column equilibrated in buffer A (to assess the intrinsic stability of the complex), or were supplemented with ATP to 2 mM for 10 min, then gel filtered. For all experiments, pyrene fluorescence was detected with an in-line fluorescence detector with excitation at 339 nm and an emission long-pass filter centred at 470 nm.

Production of mixed-ring complex and GroES binding. Mixed-ring complex MR2 was produced and purified as described previously for MR1 (ref. 21), with incubation for at least 1 h of the parent tetradecamers, D398A and 3-7-9 in MgATP at 42 °C. For studies of GroES binding to MR2, ³⁵S-GroES (40,000 c.p.m. pmol⁻¹) was incubated at a final concentration of 0.3 μ M with 1 μ M MR2 and either ATP or ADP (1 mM) in 10 mM KCl, 25 mM Tris-HCl, pH 7.4, 1 mM MgCl₂. After 20 min at 20 °C, SR398 was added to 2 μ M and, where indicated, ATP was added to a final concentration of 10 mM. After 5 min further incubation, the mixtures were chromatographed on a G4000SW_{XL} column in the same buffer containing 1 mM ADP. Fractions were collected and counted directly.

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DNA renaturation activity of the SMC complex implicated in chromosome condensation

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Chromosome condensation occurs in mitosis before the separation of sister chromatids, and requires DNA topoisomerase II (refs 1, 2) and a group of proteins called SMCs^{3–5}. The resulting condensed chromosomes in metaphase have a complex hierarchical structure^{6,7}. SMCs, the components of condensed chromosomes, are also required for the separation of sister chromatids and gene dosage compensation, and are found in a range of organisms from yeasts to mammals^{8–13}. However, the mechanisms by which the SMCs contribute to chromosome condensation are unknown. We have studied chromosomes in fission-yeast SMC mutants *cut3-477* and *cut14-208* (ref. 9), which remain largely non-condensed during mitosis at the restrictive temperature (36 °C)⁹. To test their role in DNA condensation, we isolated the proteins Cut3 and Cut14 as an oligomeric complex, and tested their interactions with isolated DNA. The complex efficiently promoted the DNA renaturation reactions (the winding up of single-strand DNAs into double helical DNA) as much as ~70-fold more efficiently than RecA¹⁴, which is a bacterial protein with similar activity. The activity of the mutant complex was heat sensitive. As DNA winding by renaturation is a potential cause of supercoiling, the SMC complex may be implicated in promoting the higher-order DNA coiling found in condensed chromosomes.

To obtain isolated Cut3 and Cut14 proteins from *Schizosaccharomyces pombe* cells, six-histidine tags were attached and they were simultaneously overproduced using the inducible promoter REP1 (ref. 15) (Fig. 1a). Cells were collected and lysed, and the extract put over a Ni-NTA column¹⁶. Fractions containing Cut14-HA6His (relative molecular mass 137,000 (*M_r* 137K)) and Cut3 (155K) at 80% purity were detected, indicating that the two proteins formed a complex¹⁰ in overproduced cells (Fig. 1b, c). The complex was further purified by phosphocellulose P11.

After sucrose-gradient centrifugation¹⁷, a broad distribution of Cut3-Cut14 at equal proportions was obtained at around 7–13S (the peak was at 13S; Fig. 1d, top). Gel filtration showed that the complex had an *M_r* of 600K or larger (data not shown). The purified Cut3-Cut14 complex thus seemed to exist as a heterotetramer or possibly a larger complex, but a smaller form (heterodimer) might also exist. A similar oligomeric complex was also isolated from extracts of cells that were not overproduced (Fig. 1d, bottom).

The purified complex was found to renature DNA extremely efficiently (Fig. 2). Denatured, single-stranded linear DNAs were mixed with the complex, proteins were extracted, and the resulting

DNA run in agarose electrophoresis¹⁸. Similar reactions were performed using RecA¹⁴ as a control. The single-stranded DNAs rapidly formed duplex DNA (double stranded) in the presence of Cut3–Cut14 complex (Fig. 2a, lanes 6–10), whereas duplex formation was minimal in the absence of complex (lanes 1–5). The double-stranded band intensity became maximal 1 min after the substrate and the complex were mixed (lane 7). DNA renaturation in the presence of RecA (a gift from T. Horii) was 10-fold slower than in the presence of the Cut3–Cut14 complex even at a sevenfold increased molar concentration (lanes 11–15), suggesting that the Cut3–Cut14 complex was ~70-fold more efficient than RecA in DNA renaturation. This was confirmed by quantitative analysis of DNA renaturation¹⁴ using radiolabelled single-strand DNA, followed by S1 digestion to remove non-renatured DNA (Fig. 2c). Renaturation of control single-stranded DNA was minimal. In contrast to RecA¹⁴, this renaturation activity did not require ATP, nor was it inhibited by analogues of ATP (data not shown). DNA renaturation activity was reported to be present in other classes of SMC subunits (similar to budding yeast Smc1 (ref. 8) and Smc3 (ref. 5)) of the calf-thymus recombination complex, which contained DNA polymerase- ϵ , DNA ligase III and endonuclease as additional subunits¹⁹.

High-molecular-weight DNAs¹⁸ accumulated in the wells were formed relatively slowly (Fig. 2a). They were not aggregates of DNA and protein, as they remained after treatment with proteinase K and phenol extraction (data not shown), but were sensitive to S1 digestion (Fig. 2b), resulting in an increase in the band intensity of double-stranded DNA. These DNAs therefore probably contained both single-stranded and double-stranded regions.

We investigated the renaturation activities of individual Cut3 and Cut14 proteins (Fig. 2d). Cut3 and Cut14 were each isolated from cells and purified by affinity chromatography (Fig. 2d, top) as oligomeric soluble forms. The renaturation reaction of purified Cut3 or Cut14 subunits was exceedingly slow (Fig. 2d, bottom: lanes

9–12 for Cut3; lanes 13–16 for Cut14), and was only slightly faster than self-annealing of single-stranded DNAs (lanes 1–4). When the Cut3 and Cut14 subunits were mixed (at 26 °C for 10 min; complex formation was verified by gel filtration) and allowed to react with the single-stranded DNAs, highly efficient renaturation activity indistinguishable from that of the Cut3–Cut14 complex was restored (Fig. 2d, lanes 17–20).

We investigated whether the renaturation activity we observed was relevant to the *in vivo* role of the Cut3 and Cut14 proteins. We began by determining the mutation sites for *cut3-477* and *cut14-208* that affect their activity *in vivo*. The SMC proteins share motifs of the terminal globular and the central putative coiled-coil regions. The N-terminal domain contained the putative nucleotide-binding sequence, whereas the C terminus had the DA box predicted to have a role for DNA binding⁸ or NTP binding¹¹. Surprisingly, the temperature-sensitive mutations resided in the coiled-coil regions (Fig. 3a). Substitution by helix-breaking proline should lead to unstable coiled-coil structures. The mutant complex was purified from cells simultaneously overproducing wild-type Cut3 and mutant Cut14, and had full activity at 26 °C (Fig. 3b, lanes 5–8). After brief heat treatment at 42 °C, the renaturation activity of the mutant complex was greatly reduced (lanes 13–16) compared to the wild-type complex (lanes 9–12). To confirm the heat sensitivity of the mutant complex, radiolabelled single-stranded

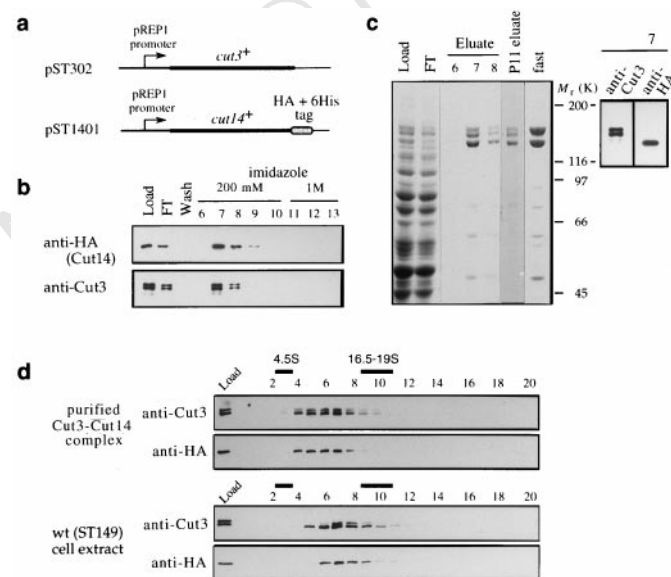


Figure 1 Identification of the Cut3–Cut14 complex. **a**, Plasmids used for overexpression. **b**, Elution of *S. pombe* extracts through the Ni-NTA column. Immunoblotting was done for the eluates. FT, flow-through fractions; HA, haemagglutinin. **c**, Left, Coomassie-blue staining patterns of the fractions. The eluates by 200 mM imidazole contained the bands at 155K and 137K (limited proteolysis occurred in Cut3 but not in the fast preparations). Right, immunoblot of fraction 7 using anti-Cut3 and anti-HA antibodies. **d**, Sucrose gradient centrifugation patterns of the isolated Cut3–Cut14 complex (top) and the extracts of wild type (wt) integrated with the *cut14+* gene tagged with HA (bottom).

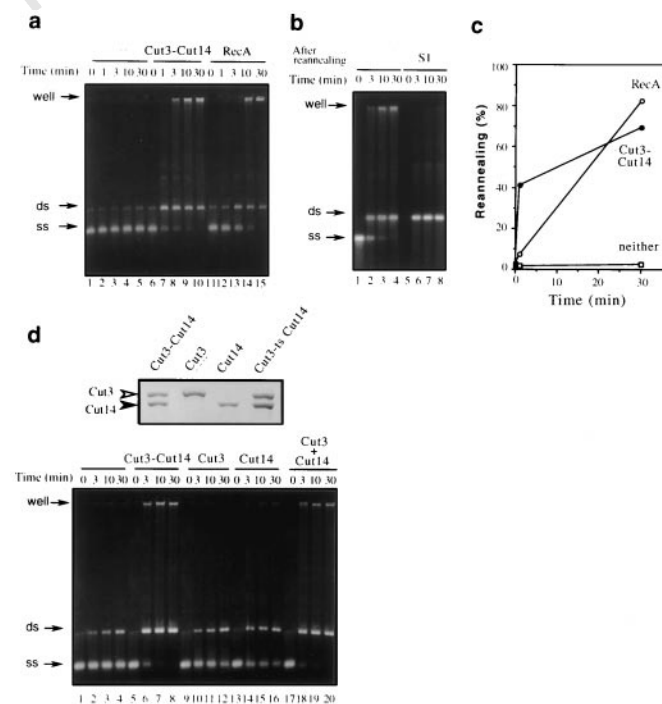


Figure 2 The Cut3–Cut14 complex contains DNA renaturation activity. **a**, Lanes 1–5, linearized, heat-denatured DNA was incubated in buffer A for 0–30 min at 30 °C, then run in agarose electrophoresis after treatment with 0.8% SDS. Lanes 6–10 incubated in the presence of Cut3–Cut14. Lanes 11–15, incubated in the presence of RecA. ss, ds and well indicate single-stranded, double-stranded and high-molecular-weight DNAs, respectively. **b**, The same reactions were done, and resulting DNAs were incubated with (lanes 5–8) or without (lanes 1–4) S1 nuclease. **c**, Radiolabelled ss DNAs were prepared and reannealed in the presence of the Cut3–Cut14 complex and RecA, followed by S1 digestion¹⁴. The amounts of renatured DNA were measured by a BioRad Molecular Imager GS-250 after autoradiography. **d**, Top, Coomassie blue stain of the individual subunits Cut3 or Cut14, and the complex of Cut3 and mutant temperature-sensitive (ts) Cut14 (used in Fig. 3). Bottom, renaturation assay: the isolated complex (lanes 5–8), individual subunits (lanes 9–12 for Cut3, 13–16 for Cut14), and the mixed subunits (lanes 17–20).

DNA was incubated with complex and the degree of reannealing quantified (Fig. 3c). At 42 °C, reannealing of the mutant complex was much slower than of the wild-type complex. These results established that the mutation in Cut14 protein induced heat-sensitive renaturation activity, probably caused by a structural aberration in the coiled-coil region. Note that the coiled-coil region is very long (~100 nm), and may facilitate duplex DNA formation or stabilize the duplex structure by interaction along the strands.

To determine whether the gross structure of nuclear chromatin may be altered in the mutant cells, we digested it with micrococcal nuclease. No obvious change was found in the nucleosome ladder patterns for nuclear chromatin²⁰ of the *cut14* mutant at 36 °C for 0 and 2 h (Fig. 4b). Nucleosome arrays seemed to be maintained. We then examined digestion patterns obtained with S1 or mung-bean nuclease, and found a significant difference between wild-type and mutant cultured at 36 °C (Fig. 4a). DNAs extracted after digestion of nuclear chromatin with S1 revealed smear patterns for *cut14* (Fig. 4a, lane 3), a weak one for *cut3* (lane 2), and hardly any for the wild type. Mung-bean nuclease also cleaved chromatin DNA in the *cut14* mutant (lane 5). In contrast, DNAs extracted first and then digested with S1 did not show such differences between wild type and *cut14* (lanes 6–9). We interpret these results to mean that unusual DNA sites, possibly with local strand separation or aberrant conformations sensitive to S1, were present in mutant chromatin but not in naked mutant DNA. Further work is necessary to identify the S1-sensitive structures.

Does the renaturation activity reported in this study contribute to chromosome condensation? Our evidence supports a correlation between the *in vitro* activity and *in vivo* condensation: *cut14* mutation results in severe defects in DNA renaturation *in vitro* and condensation *in vivo*. The fact that the activity is not produced by individual subunits explains why mutation in either *cut3* or *cut14* leads to defects in chromosome condensation⁹. However, because

activity does not require ATP, this activity is probably only a part of the total activity required for chromosome condensation. The 13S condensin, which promotes chromosome condensation in frog extracts, has been shown to contain three more subunits in addition to the Cut3 and Cut14 homologues, XCAP-C and XCAP-E²¹. The 8S condensin alone, which contains only XCAP-C and XCAP-E, hardly promotes condensation. ATP-dependent activity may be detected in the larger complex. This hypothesis is supported by the finding that the complex between SbcC (an SMC-like bacterial protein) and SbcD has ATP-dependent nuclease activity, whereas SbcD alone has nuclease activity that is independent of ATP²². Alternatively, the ATP-dependent activity can only be found in the complex obtained from mitotic cells²¹. Another possibility is that ATP is required to interact with unidentified DNA substrates with specific configurations. Although it remains to be determined how the renaturation activity actually contributes to condensation, we presume that the Cut3–Cut14 complex regulates the higher-order supercoiling, which is a characteristic feature of condensed chromosomes^{6,7}. We propose that SMC-led winding of the DNA strands is a potential cause for higher-order supercoiling. The complex possibly interacts with topoisomerase or helicase and may control the degree of DNA supercoiling in chromosomes. The Cut3–Cut14 complex is not abundant (one per 8 kilobases of DNA; data not shown), but might be sufficient for specific localization (such as to axial regions of the condensed chromosome) or for catalytic activity. Barren, which interacts with both SMC and DNA topoisomerase II (refs 21, 23), may functionally link SMC with topoisomerase II. Concerted reactions by SMC, DNA topoisomerases and helicases might be the prerequisites of proper chromosome condensation. Indeed, *cut3* and topoisomerase II mutations are synthetic lethal⁹, suggesting that they cooperate in chromosome condensation. □

Methods

Plasmids. For overproduction, pST302 and pST1401 containing the *cut3*⁺ and *cut14*⁺ genes, respectively, driven by the REP1 promoter¹⁵, were introduced into the same *S. pombe* strain. *S. cerevisiae* LEU2 and *S. pombe* ura4⁺ were used as markers for plasmids. The plasmids suppressed the mutant phenotypes in the repressed condition. To overproduce Cut3 and Cut14 separately, pST307 (the *ura4*⁺ marker) and pKZ1404 (the LEU2 marker), respectively, were used. The C termini of *cut3*⁺ and *cut14*⁺ in pST1401, pST307 and pKZ1404 were given

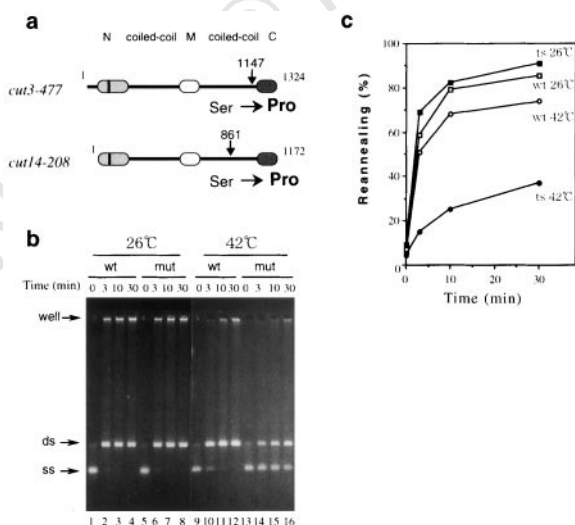


Figure 3 Heat-sensitive reannealing activity of the mutant Cut3–Cut14 complex. **a**, Mutation sites in *cut3*-477 and *cut14*-208, causing substitutions from Ser to Pro at Ser 1147 in Cut3 and Ser 861 in Cut14. Pro is known to be a helix-breaking residue. **b**, Renaturation activity of wild-type (wt) and the mutant (mut) complex. No difference in the activity was found with pretreatment at 26 °C for 10 min (lanes 1–8). If pretreated at 42 °C for 10 min, however, the activity of the mutant complex was greatly reduced (lanes 13–16) in comparison with the wild type (lanes 9–12). **c**, Quantitative analysis of reannealing by wild-type and mutant (ts) Cut3–Cut14 complex. Radiolabelled single-stranded DNA was incubated with the wild type and mutant complex after treatment at 26 or 42 °C for 10 min. The amount of renatured duplex DNA was quantified after S1 digestion.

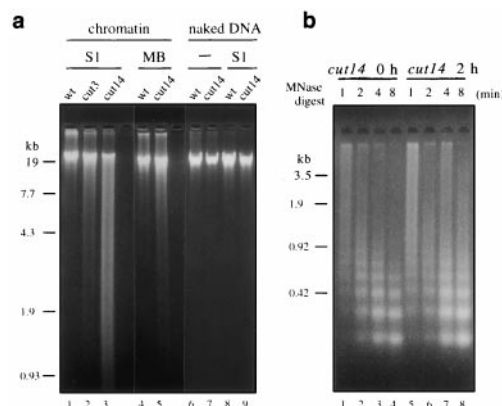


Figure 4 S1-sensitive sites in chromatin of *cut14* mutant. **a**, Preparations of nuclear chromatin²⁰ obtained from wild type and *cut3* and *cut14* mutants (cultured at 36 °C for 2 h) were incubated with S1 (lanes 1–3) or mung-bean (MB) nuclease (lanes 4, 5). DNA was then extracted with SDS and run in agarose electrophoresis. Genomic (naked) DNAs were isolated from the same cultures, and were run in agarose gel electrophoresis after treatment with (lanes 8, 9) or without S1 (lanes 6, 7). **b**, Micrococcal digestion of nuclear chromatin²⁰ from *cut14* mutant cultured at 26 °C (0 h), then transferred to 36 °C for 2 h. The patterns were shown after digestion for different time intervals.

haemagglutinin (HA) and six-histidine tags. pST1420 contained the mutant *cut14* gene.

Overproduction and protein purification. For overproduction of Cut3 and Cut14 proteins, cells carrying the plasmids described above were cultured in minimal EMM2 in the absence of thiamine for 14 h at 33 °C (for overproducing the mutant Cut14 protein, for 23 h at 26 °C). Whole-cell extracts were prepared for $1-2 \times 10^{10}$ cells. Subsequent manipulations were done at 0–4 °C. Cells were washed once in XB extraction buffer (40 mM Tris-HCl, pH 8.0, containing 0.5 M NaCl, 10% glycerol and 1 mM PMSF), disrupted by glass beads in 10 ml XB buffer, and centrifuged at 40,000 r.p.m. for 1 h using a Beckman 50.2Ti rotor. The supernatant was bound batchwise to 1 ml of Ni-NTA agarose (Qiagen)¹⁶ for 2 h. The resin was washed sequentially with 10 ml XB containing 20 mM imidazole, then 10 ml buffer N (20 mM Tris-HCl, pH 7.5, containing 150 mM NaCl, 10% glycerol, 0.02% NP-40 and 0.1 mM PMSF) containing 20 mM imidazole, and poured onto a column. After the resin was washed with 5 ml buffer N containing 20 mM imidazole, bound proteins were eluted sequentially with 5 ml buffer N containing 200 mM imidazole and then 5 ml buffer N containing 1 M imidazole. Fractions containing Cut3 and/or Cut14 were eluted by the addition of 200 mM imidazole and stored at –80 °C. Cut3 was partly cleaved during isolation procedures, but if the procedures were rapidly done in a small-scale preparation, the complex containing non-cleaved Cut3 could be obtained. This preparation also had the renaturation activity. The cellular content of Cut3 in non-overproducing wild type was determined to be 3×10^3 molecules per cell using recombinant Cut3 as standard for immunoblot, equivalent to one molecule per 8 kb genomic DNA.

DNA reannealing assay. Procedures similar to those described¹⁸ were used. Complementary DNA strands were obtained by heat-denaturing 3 kb long EcoRV-linearized pBluescript KS(+). Reaction mixtures (final volume 20 µl) contained the buffer A (20 mM Tris-HCl, pH 7.5, containing 10% glycerol, 50 mM NaCl, 10 mM MgCl₂, 1 mM ATP, 1 mM DTT), 12 µM (as nucleotides) heat-denatured DNA and the protein fractions (the Cut3–Cut14 complex, 30–60 nM; RecA, 400 nM). For ATP-free reactions, buffer B (20 mM Tris-HCl at pH 7.5, containing 10% glycerol, 100 mM NaCl, 1 mM DTT) was used instead of buffer A. Reaction mixtures were incubated at 30 °C for various lengths of time. The reaction was terminated by adding 2 µl of 8% SDS and 100 mM EDTA. Samples were analysed on 0.7% agarose gel at 1.4 V cm^{–1} for 16 h, and DNA was visualized by ethidium bromide staining.

Sucrose density-gradient centrifugation. A linear sucrose gradient (5.0 ml, 10–40%) made in HB buffer¹⁷ was overlaid with 0.1 ml Cut3–Cut14 complex solution (4.5 µg ml^{–1}) or cell extracts (equivalent to 4×10^8 cells) and run at 40,000 r.p.m. for 16 h at 4 °C. We analysed 20 fractions by immunoblot using antibodies against Cut3 or Cut14HA.

Identification of mutation sites. Approximate location of the mutation sites was determined by integration rescue. Genomic DNAs were isolated from the corresponding regions of the temperature-sensitive mutant genome, followed by nucleotide sequence determination.

Preparation of nuclear chromatin and S1 nuclease digestion. The procedures previously described for nuclear chromatin²⁰ were followed. As added 530 U of S1 or 100 U of mung-bean nuclease (TaKaRa) to 70 µl chromatin of *S. pombe* cells, incubated at 36 °C for 30 min. The reaction was terminated by 50 mM EDTA. DNAs were extracted after SDS lysis.

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Metal ion catalysis during splicing of premessenger RNA

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The removal of intervening sequences from premessenger RNA is essential for the expression of most eukaryotic genes. The spliceosome ribonucleoprotein complex catalyses intron removal by two sequential phosphotransesterification reactions¹, but the catalytic mechanisms are unknown. It has been proposed that two divalent metal ions may mediate catalysis of both reaction steps, activating the 2'- or 3'-hydroxyl groups for nucleophilic attack and stabilizing the 3'-oxanion leaving groups by direct coordination². Here we show that in splicing reactions with a precursor RNA containing a 3'-sulphur substitution at the 5' splice site, interaction between metal ion and leaving group is essential for catalysis of the first reaction step. This establishes that the spliceosome is a metalloenzyme and demonstrates a direct parallel with the catalytic strategy used by the self-splicing group I intron from *Tetrahymena*³. In contrast, 3'-sulphur substitution at the 3' splice site provides no evidence for a metal ion–leaving group interaction in the second reaction step, suggesting that the two steps of splicing proceed by different catalytic mechanisms and therefore in distinct active sites.

Substitution of an oxygen leaving group by sulphur⁴ provides a means to test metal ion–leaving group interactions because various metals differ in their ability to coordinate sulphur: for example, Mn²⁺ readily accepts sulphur as a ligand, whereas Mg²⁺ does not^{5–8}. Thus, a switch in metal specificity from Mg²⁺ to Mn²⁺ following

sulphur substitution is evidence for a direct metal ion–leaving group interaction³.

We used enzymatic ligation⁹ to synthesize an adenovirus-derived splicing substrate (Ad5-1S) containing a single 2'-deoxy-3'-thio-uridine at the 5' splice site (Fig. 1a), in which sulphur replaces the 3'-oxygen leaving group in the first step of splicing. We also prepared the standard transcribed adenovirus substrate¹⁰, as well as ligated control substrates containing a single 2'-deoxyuridine (Ad5-1D) or unmodified uridine (Ad5-1R) at the 5' splice site. The absence of a 2'-hydroxyl group at the 5' splice site is known to have no effect on the rate or accuracy of pre-mRNA splicing⁹. All four substrates were tested for *in vitro* splicing in EDTA-pretreated HeLa nuclear extract (Fig. 2a). The control adeno transcript and the ligated substrates Ad5-1R and Ad5-1D all behaved similarly: no splicing occurred in the absence of exogenous divalent metal ions (Fig. 2a, lanes 2, 6 and 10), and efficient splicing occurred in the presence of 1.5 mM MgCl₂ (Fig. 2a, lanes 3, 7 and 11) or 1.5 mM MnCl₂ (Fig. 2a, lanes 4, 8 and 12). The Ad5-1S substrate also failed to undergo splicing in the absence of exogenous divalent metal ions (Fig. 2a, lane 14); however, in contrast to the control substrates, 1.5 mM MgCl₂ was unable to support splicing (Fig. 2a, lane 15). This inhibition of splicing in MgCl₂ was apparent at all times up to 2 hours, and at MgCl₂ concentrations up to 3.5 mM (data not shown). When the splicing buffer contained 1.5 mM MnCl₂, 5'-splice-site cleavage and lariat formation occurred efficiently (Fig. 2a, lane 16), but the second step of the reaction did not. Poor nucleophilicity of sulphur at phosphate centres¹¹, which has been observed in both non-enzymatic¹² and enzymatic¹³ reactions, may be the reason for inhibition of the second step of splicing. The appearance of splicing intermediates depended on added nuclear extract (Fig. 2a, lane 17) and therefore did not simply result from incubation of the 3'-thio-modified substrate under these buffer conditions. A 2'-O-methyl oligonucleotide that sequesters the 5' end of U1 small nuclear (sn) RNA¹⁴ blocked the reaction, but a nonspecific oligonucleotide did not, indicating that the spliceosome was responsible for 5'-splice-site cleavage of the modified substrate (data not shown).

To demonstrate the accuracy of the Ad5-1S splicing reaction in MnCl₂, we synthesized a fragment of Ad5-1S pre-mRNA with a single radiolabelled phosphate adjacent to the 2'-deoxy-3'-thio-uridine residue (Fig. 2b, top). The sulphur–phosphorus bond of a

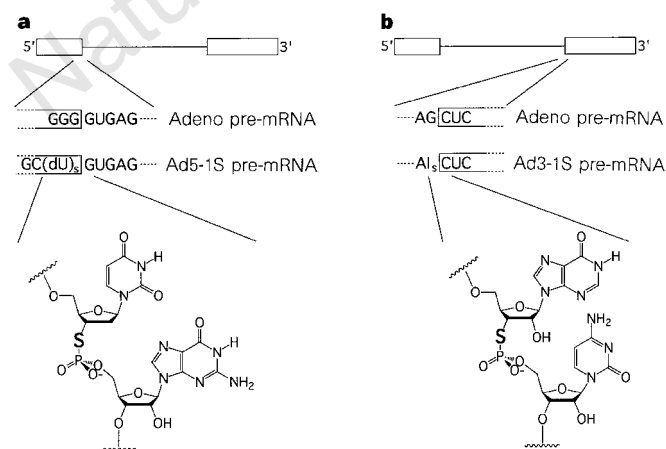


Figure 1 Modified adenovirus pre-mRNA substrates for *in vitro* splicing. The pre-mRNAs are shown schematically at the top, with splice-site sequences underneath. The structures of the nucleosides flanking the 3'-S-phosphorothiolate bond are shown at the bottom. **a**, In the Ad5-1S substrate, the guanosine at 5'-splice-site position-1 was changed to 2'-deoxy-3'-thiouridine, and the guanosine at position-2 to cytosine to avoid formation of a cryptic 5' splice site²⁵. **b**, In the Ad3-1S substrate, the guanosine at 3'-splice-site position-1 was changed to 3'-thioinosine.

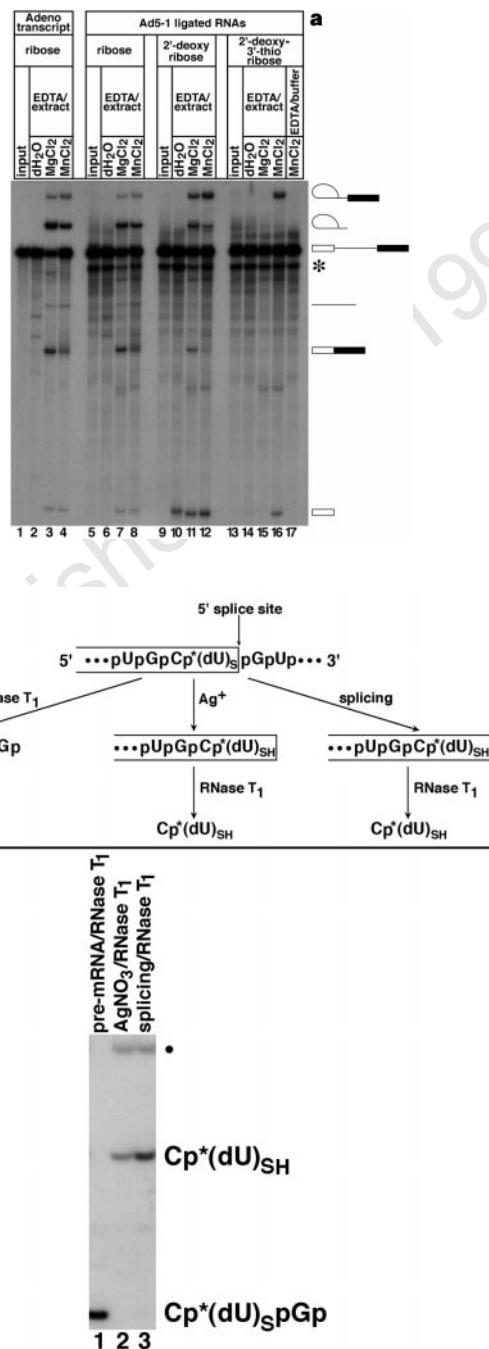


Figure 2 A 3'-thio substitution at the 5' splice site results in a metal specificity switch. **a**, HeLa nuclear extract was used for *in vitro* splicing reactions with adenovirus transcript (lanes 1–4), Ad5-1R (lanes 5–8), Ad5-1D (lanes 9–12), or Ad5-1S (lanes 13–17) pre-mRNAs. Lanes 1, 5, 9 and 13 are unspliced RNAs. Splicing reactions contained no exogenous divalent metal ions (lanes 2, 6, 10 and 14), 1.5 mM MgCl₂ (lanes 3, 7, 11 and 15), or 1.5 mM MnCl₂ (lanes 4, 8, 12, 16 and 17). In lane 17, buffer replaced nuclear extract. Splicing precursor, intermediates and products are shown schematically on the right; the asterisk denotes residual unligated 3' half RNA. With the Ad5-1D substrate, a product of slightly decreased mobility relative to exon 1 intermediate is reproducibly observed when the reactions lack exogenous divalent metal ions (lane 10). This product is not accompanied by the appearance of any lariat species, and is therefore unlikely to be related to splicing. **b**, The spliceosome accurately cleaves the sulphur–phosphorus bond of Ad5-1S pre-mRNA in the presence of Mn²⁺. The strategy for mapping the 5' splice site is shown at the top (also see text). Standards are identified on the right. The upper band in lanes 2 and 3 (indicated by a dot) may be identical to the Cp*(dU)_{SH} dinucleotide, except that the sulphhydryl group became oxidized during work-up.

3'-S-phosphorothiolate linkage can be specifically hydrolysed in the presence of dilute aqueous AgNO_3 ; normal phosphodiester linkages are unaffected^{15,16}. Ag^+ treatment combined with RNase T₁ digestion produces the dinucleotide 5'-Cp*(dU)_{SH}-3', containing a free 3'-thiol (Fig. 2b, lane 2). RNase T₁ digestion of the purified exon 1 intermediate from an Ad5-1S/Mn²⁺ splicing reaction yielded the identical 5'-Cp*(dU)_{SH}-3' dinucleotide (Fig. 2b, lane 3); iodoacetamide, which reacts specifically with sulphhydryls, quantitatively derivatized the dinucleotide to the more slowly migrating acetamide thioether¹⁶, consistent with the presence of the 3'-thiol (data not shown). These results confirm that the spliceosome cleaved the sulphur-phosphorus bond of the Ad5-1S substrate.

The inhibition of Ad5-1S splicing in MgCl_2 could arise from a failure of the spliceosomal complex to assemble, rather than from a defect in the chemical step of the splicing reaction. We monitored the assembly of splicing complexes on the Ad5-1S and Ad5-1D substrates in the presence of MgCl_2 by electrophoresis in non-denaturing gels¹⁷ (Fig. 3). Assembly of the prespliceosomal A complex, the spliceosomal B complex and the catalytically activated C complex was equally efficient with Ad5-1S and Ad5-1D (Fig. 3), even though Ad5-1S did not give rise to splicing intermediates or products in the presence of MgCl_2 (Fig. 2a, lane 15). Thus the 3'-thio modification does not interfere with spliceosome assembly in the presence of MgCl_2 , but rather blocks some subsequent step, such as catalysis of phosphotransesterification within the assembled spliceosomal active site.

If the inhibition of Ad5-1S splicing in MgCl_2 is due to a specific defect in the chemical step of the reaction, rather than being a general consequence of having the modified linkage in the vicinity of the 5' splice site, then substitution of a nearby nucleoside with 2'-deoxy-3'-thiouridine should have no effect on splicing activity. We synthesized pre-mRNAs containing uridine, 2'-deoxyuridine, or 2'-deoxy-3'-thiouridine two bases downstream of the splice site (Ad5 + 2R, Ad5 + 2D and Ad5 + 2S, respectively) in the highly conserved GU dinucleotide. All three ligated pre-mRNAs were

spliced as efficiently as the transcribed control pre-mRNA (data not shown), indicating that the inhibition of splicing in MgCl_2 is specific to 3'-thio substitution at the 5' splice junction itself. Efficient splicing of the Ad5 + 2D and Ad5 + 2S substrates also demonstrates that spliceosome assembly and splicing do not require the 2'-hydroxyl group of the U residue within the nearly invariant GU dinucleotide, as inferred from oligonucleotide *trans*-splicing reactions¹⁸.

Given that Mn²⁺ coordinates sulphur much more effectively than does Mg²⁺ (refs 5–8), the strong preference for Mn²⁺ by the 5'-splice-site-substituted precursor implies that the sulphur atom interacts with Mn²⁺ during the catalytic process. We infer that there could be an analogous metal ion-oxygen interaction during splicing of the unmodified precursor (Fig. 4). Before reaction, this interaction is expected to be weak or even repulsive¹⁹. As the scissile phosphodiester bond breaks, the 3'-oxygen develops negative charge in the transition state and the interaction with the metal ion should strengthen. An appropriately positioned metal ion in the spliceosomal active site could therefore selectively stabilize the transition state relative to the ground state, enhancing the reaction rate. Although it is not known whether protein or RNA mediates the phosphotransesterification reactions in the spliceosome, we have shown that the first step of pre-mRNA splicing occurs by a strategy that is amenable to catalysis by RNA³.

2'-Deoxy-3'-thiouridine could not be used as a probe for metal ion catalysis at the 3' splice site for two reasons: an absence of the 2'-hydroxyl group at the 3' splice site causes a 95% decrease in the efficiency of exon ligation⁹; and mutation of the highly conserved guanosine at the intron terminus to uridine abolishes pre-mRNA splicing²⁰. However, site-specific substitution of inosine for guanosine at the 3' splice site does not impair the rate or accuracy of pre-mRNA splicing *in vitro*²¹. Because an RNA dinucleotide containing 3'-thioinosine had already been characterized¹⁶, we developed a synthetic route to the phosphoramidite of 3'-thioinosine rather than 3'-thioguanosine (S.S. and J.A.P., manuscript in preparation).

We synthesized an adenovirus-derived splicing substrate (Ad3-1S) containing a single 3'-thioinosine at the 3' splice site (Fig. 1b), in which sulphur replaces the 3'-oxygen leaving group in the second step of splicing. We also prepared the standard transcribed adenovirus substrate¹⁰, as well as ligated control substrates containing a

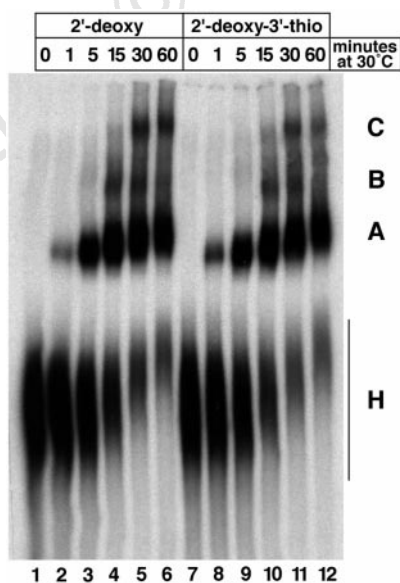


Figure 3 The 3'-thio modification does not inhibit spliceosome assembly when Mg^{2+} is present as the sole divalent metal ion. The Ad5-1D (lanes 1–6) or Ad5-1S (lanes 7–12) pre-mRNAs were spliced *in vitro* for the times indicated at the top of each lane, and splicing complexes were fractionated by electrophoresis on a non-denaturing 4% polyacrylamide gel¹⁷. The mobilities and kinetics of non-specific H complex, pre-spliceosomal A complex, spliceosomal B complex and catalytically activated spliceosomal C complex (indicated on the right) conform to previously observed patterns.¹⁷

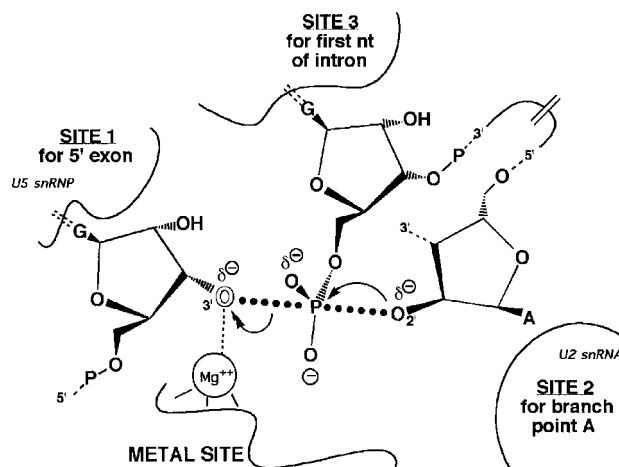


Figure 4 A catalytic metal ion is present in the spliceosomal active site for the first step of pre-mRNA splicing. The three nucleotides that participate directly in the first step of pre-mRNA splicing—the last base of exon 1, the branch point adenosine, and the first base of the intron—are shown bound to sites 1, 2 and 3 within the spliceosomal active site². A catalytic magnesium ion is inferred from this work to bind in the spliceosomal active site and directly coordinate the 3'-oxygen leaving group in the proposed transition state. Adapted from ref. 2.

guanosine (Ad3-1G) or inosine (Ad3-1I) at the 3' splice site, and tested all four substrates for *in vitro* splicing in EDTA-pretreated HeLa nuclear extract (Fig. 5). No splicing occurred in the absence of exogenous divalent metal ions (Fig. 5, lanes 2, 6, 10 and 14). As expected, efficient splicing occurred with the control adeno transcript and the Ad3-1G and Ad3-1I ligated substrates in the presence of either 1.5 mM $MgCl_2$ (Fig. 5, lanes 3, 7 and 11) or 1.5 mM $MnCl_2$ (Fig. 5, lanes 4, 8 and 12). In contrast to the results obtained at the 5' splice site, however, both steps of splicing occurred efficiently with the Ad3-1S 3'-thio substituted pre-mRNA in the presence of either of the divalent metal ions Mg^{2+} or Mn^{2+} (Fig. 5, lanes 15 and 16). This result was obtained several times using different nuclear extracts and different metal ion preparations (data not shown). Treatment of Ad3-1S pre-mRNA with $AgNO_3$ (ref. 15) gave nearly quantitative conversion to the expected hydrolysis products (data not shown), confirming the presence of the 3'-S-phosphorothiolate linkage in the pre-mRNA. Sequencing of the Ad3-1 spliced products from splicing reactions carried out in either Mg^{2+} or Mn^{2+} showed that the correct 3' splice site was used in both cases (data not shown). The possibility that added Mg^{2+} displaces trace protein-bound thiophilic metal ions, freeing them to support splicing of the 3'-thio-substituted pre-mRNA, is highly unlikely; our splicing reactions contained 0.8 mM EDTA, and the EDTA stability constants of most thiophilic divalent metal ions are at least five orders of magnitude greater than that of Mg^{2+} (ref. 22). Hence, almost all released thiophilic metal ions would have been chelated by EDTA. Furthermore, such a displacement effect might have resulted in splicing of the 5'-splice-site-substituted pre-mRNA in Mg^{2+} , which we did not observe (Fig. 2a, lane 15). We conclude that the sulphur leaving group in the exon ligation reaction does not require the presence of a metal ion to which it can coordinate effectively.

A switch in metal specificity could be masked because the chemical step of exon ligation may not be rate-limiting; however, it seems likely that disruption of an important metal ion interaction would have reduced the efficiency of splicing. For example, in the case of the Klenow fragment, there is structural evidence for a metal

ion-leaving group interaction at the 3'-5' exonuclease active site²³, and thio substitution of the leaving group decreases k_{cat}/K_M by ~50,000-fold in the presence of Mg^{2+} (J. F. Curley, C. M. Joyce, and J.A.P., manuscript submitted). Alternatively, there may be no interaction between a metal ion and the leaving group in the second chemical step of splicing. The spliceosome may stabilize the leaving group by some means other than that observed in the first reaction step, such as general acid catalysis and/or hydrogen-bond donation: the source of this proton or hydrogen-bond donor could be a metal-bound water molecule, an RNA residue with a locally perturbed pK_a , or a spliceosomal protein side chain. The first and second reaction steps probably occur at distinct active sites: for example, blockage of both reaction steps by the same phosphorothioate diastereomer argues that they are not simply forward and reverse reactions at a single active site²⁴, where the same metal ion stabilizes the leaving group in the first step and activates the nucleophile in the second step². Our observation that 3'-thio substitution blocks Mg^{2+} -mediated splicing at the 5' splice site but not the 3' splice site argues against the alternative possibility that the two steps proceed as parallel reactions in a single active site, in which the same metal ion stabilizes the leaving group in both steps. □

Methods

Pre-mRNA substrates. We synthesized 5'-O-monomethoxytrityl-2'-deoxy-3'-thiouridine 3'-S-phosphoramidite as described³; synthesis of the 5'-O-dimethoxytrityl-2'-O-t-butyl dimethylsilyl-3'-thioinosine-3'-S-phosphoramidite will be reported elsewhere. All dinucleotides and oligonucleotides were synthesized on a Millipore solid-phase DNA/RNA synthesizer and deprotected and purified using standard procedures, except that 2'-deoxy-3'-thiouridine and 3'-thioinosine were incorporated as described⁴. We synthesized the following dinucleotides and oligonucleotides: 5'-(dU)₆pG-3', 5'-(dU)pG-3', Ad5-1R/21 (5'-UGUGA-GUACUCCUCUCAAAA-3'), Ad5-1D/21 (5'-(dU)GUGAG UACUCCUCUCAAAA-3'), Ad5-1S/21 (5'-(dU)GUGAGUACUCCUCUCAAAA-3'), Ad5-2-10p (5'-pCAGCUGUUGC-3'), Ad3-1G (5'-AGCUCG CGGUU-3'), Ad3-1I (5'-AICUCGCGGUU-3'), and Ad3-1S (5'-AICUC GCGGUU-3') ((dU) is 2'-deoxyuridine, (dU)_s is 2'-deoxy-3'-thiouridine, p is phosphate, I is inosine, and I_s is 3'-thioinosine).

For the experiments shown in Figs 2a and 3, we synthesized the Ad5-1 substrates as described²⁵, except that the dinucleotides UpG (Sigma), (dU)pG, or (dU)_spG were used to initiate transcription of the 3' half RNA, and the 3' half RNA was labelled internally with [α -³²P]UTP. We synthesized the Ad5 + 2 substrates as described²⁵, using the dinucleotides UpG, (dU)pG or (dU)_spG to initiate transcription of the 3' half RNA. For the experiment shown in Fig. 2b, we synthesized the Ad5-1 substrates with a single ³²P-phosphate adjacent to the modified nucleotide. The Ad5-1R/21, Ad5-1D/21 and Ad5-1S/21 oligonucleotides were 5'-³²P-phosphorylated and ligated²⁶ to the Ad5-2-10p oligonucleotide to generate the fragments Ad5-1R/30, Ad5-1D/30 and Ad5-1S/30, respectively. We then ligated these fragments to Ad5-10 5' RNA (corresponding to the first 57 nucleotides of exon 1) and Ad5 + 21 3' RNA (corresponding to the last 223 nucleotides of the intron and all 99 nucleotides of exon 2).

For synthesis of the Ad3-1 substrates, the Ad3-1G, Ad3-1I and Ad3-1S oligonucleotides were 5'-³²P-phosphorylated and ligated²⁶ to the Ad3-3 5' RNA (corresponding to exon 1 and the first 241 nucleotides of the intron) and the Ad3 + 10 3' RNA (corresponding to nucleotides 10–99 of exon 2). Before the ligation reactions, we 3'-end-labelled the Ad3 + 10 3' RNA with [α -³²P]cordycepin triphosphate using yeast poly(A) polymerase (US Biochemicals).

Nuclear extracts and *in vitro* splicing reactions. HeLa cells were purchased from the National Cell Culture Center (Minnesota), and nuclear extract prepared as described²⁷, except that dialysis buffer contained 42 mM $(NH_4)_2SO_4$ instead of KCl. For *in vitro* splicing, nuclear extract (already containing 0.2 mM EDTA) was supplemented with an additional 2.5 mM EDTA²⁸, and incubated on ice for 30 min. Splicing reactions (10 μ l) included 30% nuclear extract (final EDTA concentration 0.8 mM), 0.5 mM ATP, 0.1 mM cordycepin triphosphate, 20 mM creatine phosphate, 20 ng μ l⁻¹ carrier RNA, trace quantities of radiolabelled splicing substrate, and 1–2 units μ l⁻¹ RNA-guard RNase inhibitor (Promega). We also included water, $MgCl_2$ or $MnCl_2$, as described in the text and figure legends; metal ion chlorides (Aldrich) were >99.99%

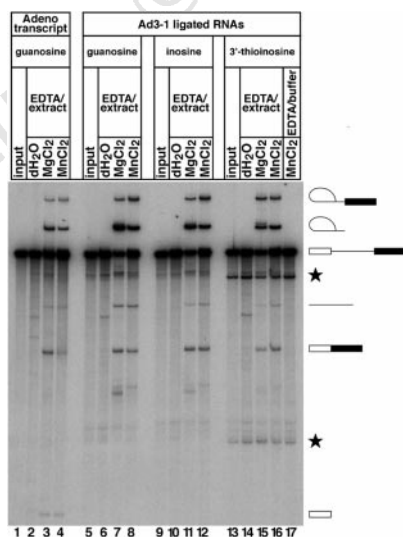


Figure 5 Mg^{2+} supports *in vitro* splicing of a pre-mRNA with a 3'-thio substitution at the 3' splice site. *In vitro* splicing reactions were carried out with adenovirus transcript¹⁰ (lanes 1–4) and Ad3-1G (lanes 5–8), Ad3-1I (lanes 9–12), and Ad3-1S (lanes 13–17) ligated RNAs as for Fig. 2a. Splicing precursor, intermediates and products are shown schematically on the right. Stars indicate background cleavage products (RNA containing an inosine 3'-S-phosphorothiolate linkage cleaves ~2,000-fold faster than unmodified phosphate-linked RNA¹⁶). For the ligated pre-mRNAs, the exon 1 intermediate contains no radiolabel and is therefore not visible.

pure. Splicing reactions were for 1–2 h at 30 °C. Extracted and precipitated RNAs were fractionated on a denaturing 8% polyacrylamide gel; the gel and electrophoresis buffer (1 × TBE) contained 10 mM dithiothreitol (DTT) and gels were pre-run for at least 6 h.

Splice-site mapping. For 5′-splice-site mapping, we included the Ad5-1S pre-mRNA in splicing reactions as described and gel-purified the 5′ exon intermediate. To generate a standard, the Ad5-1S/30 fragment was treated with AgNO₃ (ref. 15), and the 10-nucleotide labelled product of silver cleavage (5′-pCAGCUGUUGC(dU)_{SH}-3′) was purified in a 20% polyacrylamide denaturing gel containing 10 mM DTT. Following elution, we desalted the oligonucleotide in a Sep-Pac C₁₈ cartridge (Waters) and dried it *in vacuo*. The purified 5′ exon intermediate, the Ag⁺-cleaved fragment, and the Ad5-1 pre-mRNA were digested with RNase T₁ and fractionated in a 20% polyacrylamide denaturing gel containing 10 mM DTT. RNase T₁ digestions⁹ contained 10 mM DTT.

For 3′-splice-site mapping, extracted and precipitated RNAs from 100-μl splicing reactions (Fig. 3b, lanes 15 and 16) were reverse transcribed using a DNA primer (Ad.33) complementary to the 3′-terminal 21 nucleotides of the Ad3-1S substrate. We used the products of these reactions as PCR templates, with Ad.33 primer and another oligonucleotide (Ad.55) corresponding to the 5′-terminal 20 nucleotides of the Ad3-1S substrate, and sequenced the PCR products derived from ligated exons.

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A second catalytic metal ion in a group I ribozyme

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Although only a subset of protein enzymes depend on the presence of a metal ion for their catalytic function, all naturally occurring RNA enzymes require metal ions to stabilize their structure and for catalytic competence¹. In the self-splicing group I intron from *Tetrahymena thermophila*², several divalent metals can serve structural roles, but only Mg²⁺ and Mn²⁺ promote splice-site cleavage and exon ligation^{3,4}. A study of a ribozyme reaction analogous to 5′-splice-site cleavage by guanosine uncovered the first metal ion with a definitive role in catalysis. Substitution of the 3′-oxygen of the leaving group with sulphur resulted in a metal-specificity switch, indicating an interaction between the leaving group and the metal ion⁵. Here we use 3′-(thioinosyl)-(3′ → 5′)-uridine⁶, IspU, as a substrate in a reaction that emulates exon ligation. Activity requires the addition of a thiophilic metal ion (Cd²⁺ or Mn²⁺), providing evidence for stabilization of the leaving group by a metal ion in that step of splicing. Based on the principle of microscopic reversibility, this metal ion activates the nucleophilic 3′-hydroxyl of guanosine in the first step of splicing, supporting the model of a two-metal-ion active site⁷.

The self-splicing reaction performed by the *Tetrahymena* intron consists of two consecutive phosphotransesterification reactions. The mechanism of the first step, 5′-splice-site cleavage, has been studied using a truncated form of the intron (L-21 ScaI) in the forward reaction (G + CCCUCUpA → GpA + CCCUCU, where only the reactive phosphate is shown explicitly) (Fig. 1a)^{2,8}. The second step of splicing, exon ligation, is chemically the reverse of the first step⁹ and is thought to occur in the same active site^{10,11}. This step is mimicked by the reverse of the ribozyme reaction (GpN + CCCUCU → G + CCCUCUpN, where N is A, C or U) (Fig. 1a)^{12–14}. Kinetic studies of this reverse reaction have used dinucleotides with guanosine at the 5′-position. However, inosine will substitute for guanosine in both splicing and the enzymatic forward reaction^{15,16}. For ease in chemical synthesis, we chose IspU, an inosine-containing dinucleotide with a 3′-S-phosphorothiolate linkage⁶, to prospect for a metal ion interacting with the leaving group in the reverse reaction (Fig. 1b).

Under conditions previously shown to support the reverse reaction efficiently, namely pH 7.0 and high MgCl₂ concentration (110 mM)¹³, only the unmodified dinucleotide, IpU, was a substrate for transesterification (Fig. 2). Addition of Mn²⁺ (1–100 mM) resulted in progressively higher rates of transesterification of IspU (Fig. 2). To find conditions under which the observed rate constant (*k*_{obs}) reflects the chemical step, a pH profile was determined for the

reaction with IpU (data not shown). From pH 5.5 to 7.0, $\log k_{\text{obs}}$ increased linearly with pH, and slopes of 0.91 and 0.79 were measured for reactions in 20 mM MgCl_2 and in 10 mM MgCl_2 /10 mM MnCl_2 , respectively. This dependence is a hallmark for the actual cleavage event being rate-determining^{13,17}. Consequently, all subsequent reactions were done at pH 5.9. The dinucleotide binds so weakly that it is subsaturating at all accessible concentrations (data not shown). Thus, the observed rates are proportional to the kinetic parameter k_{cat}/K_m , and any difference in binding between the dinucleotides would be manifested in these measurements.

To sample a range of Lewis acids, the reaction was conducted with Mn^{2+} , Zn^{2+} , Cd^{2+} or Co^{2+} in combination with enough MgCl_2 to ensure proper folding of the ribozyme. Although high metal-ion concentration did enhance the rate of reaction, the rate constants were determined with less metal to alleviate nonspecific degradation of the RNA and to accommodate solubility limitations of the metal salts tested. As shown in Fig. 3a, IpU transesterification was catalysed by Mg^{2+} alone, and substitution of a portion of the Mg^{2+} with either Mn^{2+} or Zn^{2+} stimulated the observed reaction rate, as seen previously in measurements of the forward reaction⁵. In contrast, IspU was not a substrate when only Mg^{2+} was included in the reaction (Fig. 3b). Addition of Mn^{2+} , Cd^{2+} or Zn^{2+} restored the reaction to rates equivalent to or exceeding that for IpU with Mg^{2+}

alone. Although both Mn^{2+} and Cd^{2+} facilitated the IspU reaction more than the IpU reaction (Fig. 3), only Cd^{2+} gave a true specificity switch: that is, Mg^{2+} and Cd^{2+} afforded equivalent rates of reaction with IpU, whereas the Cd^{2+} rate greatly exceeded that for Mg^{2+} in the IspU reactions. Considering the preference of Cd^{2+} for sulphur ligands over oxygen ligands¹⁸, the metal-specificity switch observed upon changing a single atom in the substrate (3'-O $\rightarrow$ 3'-S) supports the conclusion that there is a direct interaction of the metal ion with this atom.

The concentration dependence of the Cd^{2+} and Mn^{2+} effects was explored further. The IspU transesterification rate increased with Cd^{2+} concentration up to 2 mM, whereas the reaction with the unmodified dinucleotide was unaffected over the concentration range examined (Fig. 4a). At concentrations of $\text{CdCl}_2 > 0.5$ mM, the actual rate of IspU cleavage exceeded that of IpU, an effect that was not achieved with any other metal ion. From 0–1 mM Mn^{2+} , the IspU cleavage rate increased with little effect on the IpU reaction (data not shown). Above 1 mM, increasing Mn^{2+} concentration affected the reactions with IpU and IspU to the same extent, reflecting a general enhancement of ribozyme activity.

If the IspU-ribozyme-oligonucleotide complex formed a non-native metal-ion site, exclusive enhancement of the IspU reaction might also be observed. To test this possibility, we investigated

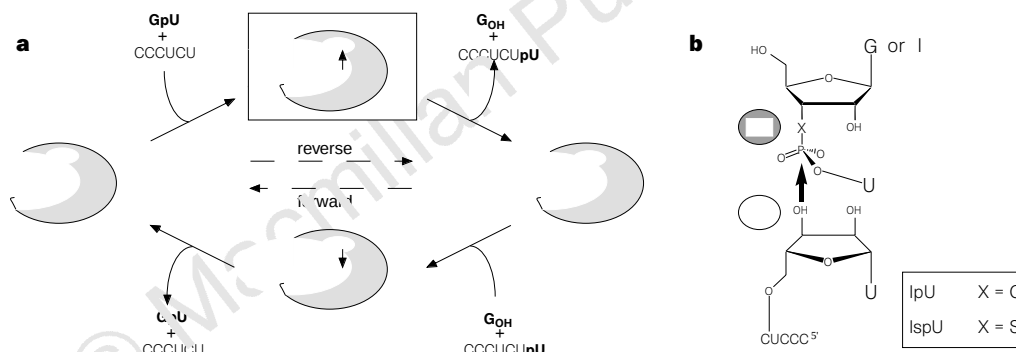


Figure 1 Ribozyme catalysis: reactions and proposed mechanism. **a**, Reactions catalysed by a truncated form of the *Tetrahymena* group I intron, the L-21 *Scal* ribozyme. The 'reverse' reaction models the second step of splicing, exon ligation. A dinucleotide, GpU, binds in the G-binding site, emulating the 3'-splice site, and an oligonucleotide (CCCUCU) added in *trans* functions as a 5'-exon mimic. Nucleophilic attack proceeds to liberate guanosine, the intact catalyst and the shortened oligonucleotide (CCCUCUpU), analogous to the ligated exons generated by self-splicing. The 'forward' reaction models 5'-splice site cleavage. A guanosine molecule binds, and its 3'-hydroxyl acts as the nucleophile to cleave

the 3'-terminal nucleotide of an added oligonucleotide (CCCUCUpU), producing a dinucleotide (GpU) and a shortened 5'-exon analogue (CCCUCU). **b**, Model for RNA-catalysed phosphodiester bond cleavage. Detail of complex boxed in **a**. The bold arrow indicates the trajectory of nucleophilic attack by the terminal 3'-hydroxyl of CCCUCU. The white ball represents the metal ion identified by Piccirilli *et al.*⁵ which interacts with the leaving group in the forward reaction; by microscopic reversibility, it interacts with the 3'-O of the nucleophile in the reverse reaction shown here. The stippled ball is the metal ion that interacts with the leaving group in the cleavage of IpU and IspU, as tested here.

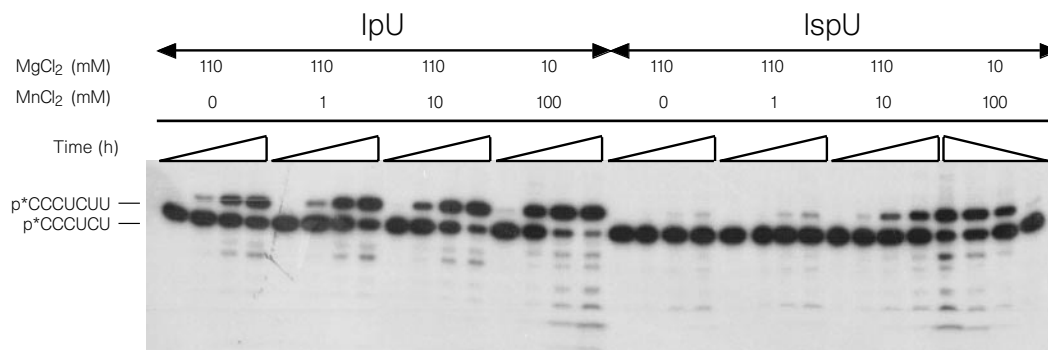


Figure 2 Cleavage of IspU requires Mn^{2+} . Scanned autoradiograph of a polyacrylamide gel showing IpU and IspU reactions as a function of time. Timepoints shown are 0.05, 2, 12 and 24 h. The dinucleotide substrates (1 mM) were reacted with 100 nM L-21 ribozyme, 1 μM CCCUCU and a trace amount (<1 nM) of p*CCCUCU at pH 7.0 in the presence of Mg^{2+} and Mn^{2+} at the indicated

concentrations. The small amount of product co-migrating with p*CCCUCUpU in the IspU lanes in the absence of Mn^{2+} was ribozyme-dependent and was also found in the absence of added dinucleotide²⁷. The ribozyme was folded in 20 mM MgCl_2 and then added to the appropriate reaction mixture.

whether Cd^{2+} was binding in a site capable of being occupied by Mg^{2+} by using MgCl_2 as a competitor of the enhanced reaction (Fig. 4b). The IspU reaction was inhibited, whereas the IpU reaction was stimulated; this latter effect has been observed previously in studies of the reverse reaction using GpA as substrate¹³. Net inhibition by Mg^{2+} is seen in the ratio of the observed rates for IspU and IpU transesterification (diamonds in Fig. 4b), consistent with a common binding site for Cd^{2+} and Mg^{2+} . The Mn^{2+} -enhanced reaction was also inhibited by Mg^{2+} (data not shown), as previously shown for the forward reaction⁵. Apparently, the thio-substituted catalytic site still binds Mg^{2+} , but the bound metal ion is not effective in catalysis.

The effect of metal ions in solution on reactions with sulphur leaving groups has been examined for intramolecular cleavage of nucleoside-(3' → 5')-5'-thionucleotide substrates ($\text{NpsN}' \rightarrow \text{N} > \text{p} + \text{sN}'$, where N is C or U and N' is A or U). It has been found that the rate of cleavage of d(ACGGTCT)rCpsd(ACGAGC) increases with 'softness' of the added metal ion¹⁹ or alternatively that cleavage of UpsU is unaffected by addition of either Zn^{2+} or Cd^{2+} relative to cleavage of unmodified phosphodiester²⁰. The preference for the catalytic metal ion in ribozyme-mediated IspU cleavage ($\text{Cd}^{2+} > \text{Mn}^{2+} > \text{Zn}^{2+} > \text{Co}^{2+} = \text{Mg}^{2+}$) stands in contrast to these results for uncatalysed cleavage, supporting the conclusion that a distinct site participates in catalysis.

Additionally, the metal-ion preference observed here for the reverse reaction differs from that determined for cleavage of d(CCCUCUspAAAAA) in the forward reaction ($\text{Zn}^{2+} > \text{Mn}^{2+} > \text{Cd}^{2+} > \text{Co}^{2+} > \text{Mg}^{2+}$)⁵. This would be difficult to explain if the thio substrates were merely recruiting thiophilic metal ions from solu-

tion, but it is what would be expected if the ribozyme were to provide two discrete binding sites for the two catalytic metal ions, each with its own ligand environment.

As shown here, the metal ion (stippled ion in Fig. 1b) stabilizing the leaving group during the reverse reaction would, by microscopic reversibility, be interacting with the nucleophile (3'-hydroxyl of G or I) in the forward reaction. These two RNA enzyme reactions correspond to the exon ligation and 5'-splice-site cleavage steps of self-splicing, respectively. Conversely, the metal ion (white ion in Fig. 1b) stabilizing the leaving group in the forward reaction must be interacting with the nucleophile in the reverse reaction³. Kinetic evidence from metal-ion competition experiments also supports a model featuring two ions that are important for the chemical step²¹. Because the sulphur-substitution experiments were designed to look for metals in proximity to particular atoms, additional interactions (such as with the 2'-O of G; ref. 22) may not have been detected. Two-metal-ion mechanisms have been explored as general solutions for how protein and RNA enzymes catalyse phosphoryl transfer⁷. The *Tetrahymena* group I ribozyme now provides the first example of an RNA enzyme that uses such a mechanism. □

Methods

Polyacrylamide gel electrophoresis (PAGE). Oligonucleotides were separated on 20–24% polyacrylamide gels for both preparation (non-denaturing) and analysis (denaturing, 7 M urea). Analytical gels were dried and then analysed using a Molecular Dynamics PhosphorImager.

RNA preparation: oligonucleotides and ribozyme. Ribo-oligonucleotides, CCCUCU and CCCUCUU, were synthesized, deprotected and purified using

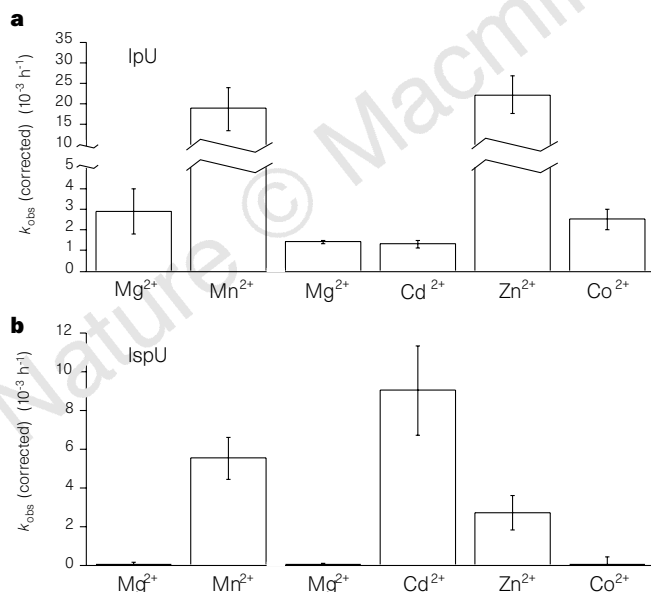


Figure 3 Metal specificity of reactions containing IpU and IspU. **a**, Reaction with the unmodified dinucleotide IpU was stimulated above the Mg^{2+} -only rate by Mn^{2+} and by Zn^{2+} . **b**, IspU reacted only in the presence of thiophilic metal ions. Reactions were carried out using 18 mM MgCl_2 and 2 mM of the indicated divalent metal chloride salt, apart from reactions with MnCl_2 which contained 10 mM MgCl_2 and 10 mM MnCl_2 . First-order rate constants (k_{obs}) were determined from initial rates (typically 4–8 timepoints) at pH 5.9 and 40 °C with 0.5 mM dinucleotide, and represent an average of two (MnCl_2) or three (others) independent experiments. Values are corrected by subtracting the rate of formation of p*CCCUCUU in the absence of added dinucleotide. Reactions containing IpU, IspU and no added dinucleotide were done in parallel using the same stocks of folded ribozyme and metal ions. Errors shown are calculated standard errors. Two values are shown for MgCl_2 : first bar, values determined in parallel with MnCl_2 experiments; and third bar, values determined in parallel with ZnCl_2 , CdCl_2 and CoCl_2 .

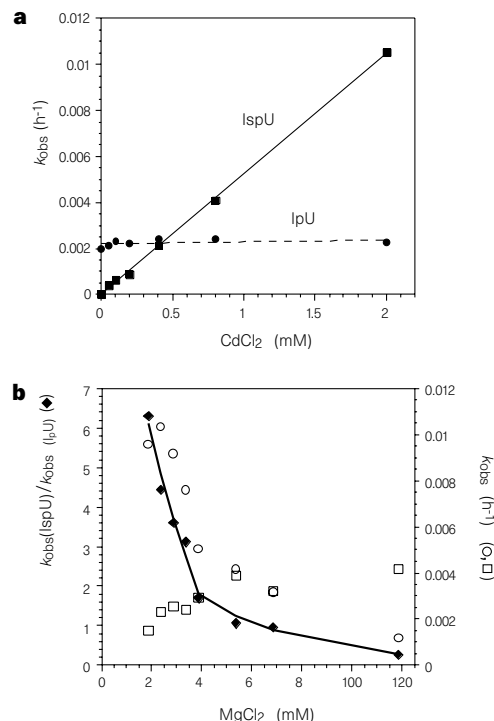


Figure 4 Cd^{2+} interacts in the active site. **a**, CdCl_2 (0–2 mM) accelerated the rate of transesterification of IspU; the rate for IpU was unaffected. At higher CdCl_2 concentrations (4 and 8 mM), both IpU and IspU cleavage reactions were inhibited (data not shown). The line represents a linear fit (Kaleidagraph) to the data obtained at pH 5.9 with 0.5 mM dinucleotide. The total divalent-metal concentration was maintained at 20 mM by adjustment of $[\text{MgCl}_2]$. **b**, Mg^{2+} competes for Cd^{2+} binding. Addition of MgCl_2 (0–100 mM) to a reaction containing 18.5 mM MgCl_2 and 2 mM CdCl_2 inhibited cleavage of the modified dinucleotide. Right axis, individual rate constants for cleavage of IspU (circles) and IpU (squares); left axis, ratio of these rate constants (diamonds). Reactions were buffered at pH 5.9 and contained 0.5 mM dinucleotide.

standard procedures¹⁶. Oligonucleotides were 5'-end labelled with T4 polynucleotide kinase and [γ -³²P]ATP (for example, CCCUCU $\rightarrow$ p*CCCUCU; where p* indicates the radioactive phosphorus). L-21 *ScaI* ribozyme was prepared by transcription from the *ScaI*-cut plasmid pT7L-21 and was purified as described²³.

Dinucleotide preparation: IpU and IspU. Inosyl-(3' $\rightarrow$ 5')-uridine (IpU) was purchased from Sigma and used without further purification. 3'-(Thioinosyl)-(3' $\rightarrow$ 5')-uridine triethyl ammonium salt (IspU) was synthesized as described⁶ with the following differences. The previously described 2',3'-anhydroinosine²⁴ was silylated using *t*-butyldiphenylsilyl chloride (1.1 mol equiv) in dry pyridine (room temperature for 72 h, then 60 °C for 24 h) to give 5'-*O*-*t*-butyldiphenylsilyl-2',3'-anhydroinosine (75%). (A 5'-silyl protecting group was also used in the synthesis of the 5'-*O*-dimethoxytrityl-2'-*O*-*t*-butyldimethylsilyl-3'-thioinosine-3'-S-phosphoramidite used in ref. 25). Subsequent conversion to 9-[5'-*O*-(*t*-butyldiphenylsilyl)-3'-deoxy-3'-iodo- β -D-xylofuranosyl]hypoxanthine was achieved in 70% yield by reaction with sodium iodide (4 mol equiv) and incremental addition of boron trifluoride-etherate (3.5 mol equiv) over 48 h in dry acetonitrile at 0–4 °C. Synthesis then progressed as described⁶, but using 5'-*t*-butyldiphenylsilyl intermediates in place of the 5'-trityl-protected compounds. Silyl-protected IspU was deblocked by treatment with NEt₃·HF (triethylamine tris-hydrofluoride) and purified by chromatography on DEAE-Sephadex (elution with 0 to 0.5 M triethylammonium bicarbonate), followed by flash chromatography on octadecylsilyl silica (elution with 2% methanol in water). The product was identical in every respect to the previously prepared sample.

Ribozyme reactions. The ribozyme was preincubated with MgCl₂ in NaMES buffer (pH 5.9 or 7.0) for 20 min at 50 °C (ref. 26). This ribozyme-metal mix was combined with the substrate oligonucleotide and incubated at 40 °C for 5 min. Reactions were initiated with the addition of the appropriate dinucleotide or an equal volume of H₂O and incubated at 40 °C for the duration of the experiment. Reactions were followed for 8–26 h. A typical reaction included: 50 mM NaMES, pH 5.9, 20–100 mM MgCl₂, 100 nM L-21 RNA, trace p*CCCUCU, 1 μ M CCCUCU and 0.5–1 mM dinucleotide. In experiments where either the concentration of MgCl₂ or the divalent ion was varied, the ribozyme was preincubated in 10–20 mM MgCl₂ and then diluted into a reaction mixture containing the additional metal components and the substrate oligonucleotide. Divalent metal salts were $\geq$ 99.99% pure. ZnCl₂, CdCl₂ and CoCl₂ were stored as 20 mM stock solutions in 50 mM NaMES, pH 5.9. Over the course of an experiment, reactions were sampled 4–8 times by removing aliquots of 1.5–2 μ l which were quenched by addition to 2.5 volumes of stop solution. The final concentration of EDTA in the stop solution was adjusted to ensure >2 -fold excess of EDTA over the total divalent metal ion concentration.

Kinetics calculations. First-order rate constants (k_{obs}) were determined from initial rates. Values in Fig. 3 were corrected by subtracting the rate of product formation in the absence of added dinucleotide; rates in Fig. 4 were uncorrected. Previous work examining a metal-specificity switch⁵ summarized metal ion effects as “relief of the thio-effect”, defined as a ratio of rate constants: $[k(3'O)/k(3'S)]^{\text{metal}1}/[k(3'O)/k(3'S)]^{\text{metal}2}$. We have avoided this approach here because some of the $k(3'S)$ values are close to zero and have large uncertainties: division then amplifies the uncertainty to an unacceptable level.

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correction

CO₂ fixation and photoevolution of H₂ and O₂ in a mutant of *Chlamydomonas* lacking photosystem I

E. Greenbaum, J. W. Lee, C. V. Tevault, S. L. Blankinship & L. J. Mets

Nature **376**, 438–441 (1995)

We reported in this paper and elsewhere¹ that mutants of the green alga *Chlamydomonas reinhardtii* that lacked detectable levels of functional photosystem I (PSI) are capable of photoreduction of atmospheric CO₂, autotrophic growth, and sustained simultaneous photoevolution of H₂ and O₂. Although the absence of PSI in mutants B4 and F8 has been confirmed by physical, biochemical and genetic techniques, subsequent analyses in our own laboratories, as well as in those of colleagues to whom we have sent the mutants, indicate that there is variability in the PSI content of the cultures depending on growth conditions. Although some strains retain undetectable levels of P700, others contain variable (0–20%) amounts of wild-type P700. (See also refs 2, 3).

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3. Greenbaum, E., Lee, J. W., Tevault, C. V., Blankinship, S. L. & Mets, L. J. *Nature* 379, 305(1996). [Links](#)



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Molecular means and methods

Items on view at the meeting of the American Society for Biochemistry and Molecular Biology in San Francisco may include a versatile hybridization oven, affinity purification reagents and a dual-laser-induced fluorescence detector.

Brandel FR115 microfractionator

From Semat

An automatic, programmable clinical fractionator

This microfractionator should provide the precise amounts of solution that need to be extracted from centrifugally banded solutions for clinical analysis. It can collect aliquots as small as 0.25 µl from centrifuge tubes without mixing the tube's contents. Applications of this technology include DNA fractioning of LipoProdrines, or the quantitative characterization of macromolecules. The FR115 will accept standard cylindrical 1.0-ml and 0.2-ml polycarbonate centrifuge tubes, as well as 0.15-ml capacity, high-precision quartz tubes. In addition, a recently introduced block assembly will accept the 5.1-ml, TLA 100.4 tube used with Beckman's rotor TL100 machines, and is interchangeable with the supplied assembly. The use of an open design in the FR115 allows adaptations for special applications and additional tube types to be incorporated easily. The size and number of fractions, the collection rate and all other variables are user adjustable through the programmable keypad. Individual fractions can be collected in liquid form, with or without chase or scintillation fluid, using an outlet capillary, or they can be deposited directly onto glass-fibre filters. The instrument can be employed manually in single-step operation, using a panel button or foot pedal, or automatically in a repetitive mode, using an external pump and fraction collector.

Reader Enquiry No. 100



The FR115 automatic microfractionator.



Vision 2000 MALDI MS for DNA sequencing.

Vision 2000

From Thermo BioAnalysis

A system for DNA sequencing that uses MALDI mass spectrometry

According to the manufacturer, this spectrometer has been used to read DNA sequencing ladders generated by solid-phase Sanger sequencing protocols. Conventional DNA sequencing using Sanger chemistry employs gel electrophoresis to separate specifically terminated DNA fragments within the four-base sets. In methods that use MALDI MS, DNA is immobilized onto magnetic beads using biotin-streptavidin chemistry. After sequence ladder generation, the beads are placed directly onto a Vision 2000 sample target for analysis. The methods, developed by Hubert Köster, are said to demonstrate the potential for increasing throughput in DNA sequencing by several orders of magnitude with improved accuracy, reliability and higher base identification.

Reader Enquiry No. 101

Spin'n'Stack oven

From Hybaid

For whole-mount in situ hybridization

The 'tilting wheel' accessory provided with the oven enables whole-mount samples to be rotated at speeds between 5–20 r.p.m. and at adjustable tilt angles between 0° and 180°. These two provisions allow for the use of minimal solution volumes and provide the gentle mixing action required for fragile whole-mount samples. In conjunction with the accurate temperature control and uniformity provided by the oven, suitable conditions can be created for whole-mount *in situ* protocols. Tube carousels are available to hold a variety of tube types from 0.5-ml microcentrifuge tubes through to 50-ml tissue culture tubes. These double as tube racks to enable easy benchtop manipulations and sample storage. The oven has been designed to be fully compatible with the existing Shake'n'Stack oven accessories,

so that a range of hybridization rotisseries and a shaking platform can be used if desired.

Reader Enquiry No. 102

Gels analysis and separation

Hi-Lo DNA marker

From BioNexus

A marker that covers the range from 50 bp to 10,000 bp

According to the manufacturer, the all-purpose Hi-Lo DNA marker is easy to read with several landmarks and logically spaced bands. The marker is informative, allowing accurate sizing of DNA bands over its entire range. This is said to eliminate the need to purchase two separate markers. It is stable at room temperature, allowing shipping and storage at ambient temperature.

Reader Enquiry No. 103

Speedlight Platinum GDS

From Lighttools Research

A workstation designed from the ground up for gel documentation

This system incorporates on-chip integration for very low light level imaging, digital storage to disk for long-term archival and post processing, time/date/ID and text for annotation, as well as digital background subtraction, sensor lock for 'sharp' prints, and camera saturation detection to keep images in the camera's linear dynamic range. Users can access all of these functions, according to the manufacturer, through a rugged, hand-held controller. The design philosophy is for centralized gel documentation and off-line, decentralized



A gel documentation system, Lighttools Research.

data processing to eliminate the gel documentation system from becoming a data processing bottleneck.

Reader Enquiry No. 104

EKAway

From Invitrogen

Enterokinase removal using affinity resin

Many protein purification strategies incorporate affinity tags for purification by affinity chromatography. Of these, a number also contain enterokinase cleavage sites for the removal of the affinity tag by treatment with enterokinases, for example EKMax. However, in order to obtain high-purity recombinant protein it is often necessary to remove the contaminating enterokinase itself. EKAway is an enterokinase affinity resin designed specifically for this purpose and is stated to remove greater than 99 per cent of the enterokinase from the protein/enterokinase mixture. It is also said to be effective in removing other trypsin proteases.

Reader Enquiry No. 105

Oligo-dT₃₀ particles

From Scigen

A range of affinity purification reagents, including paramagnetic oligo-dT₃₀ particles for rapid mRNA separation

The magnetic Oligo-dT₃₀ particles are said to be well suited for isolating mature eukaryotic mRNA from crude RNA preparations or cell lysis extracts for a cost that is stated to be 3–7 times less than other oligo-dT particles. Each Oligo-dT₃₀ is covalently coupled via a 5' spacer to enhance specific binding at high stringency and minimize steric hindrance. The density of the 3-µm particle has been optimized for mRNA isolation, providing easy mixing with fast magnetic sedimentation.

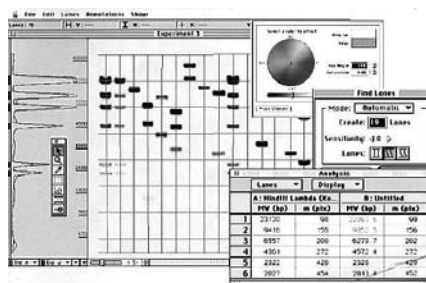
Reader Enquiry No. 106

1D image analysis software

From Eastman Kodak

Version 2.0 of the company's one-dimensional image analysis software for Macintosh and Windows

The software acquires digital images using Kodak's Digital Science cameras or any TWAIN compliant scanner, and is well suited for analysing DNA, RNA and protein electrophoresis gels and blots. Analysis data includes mass, molecular weight, intensity and mobility values. This new version offers users several new features, including automated lane finding, non-destructive annotations, Gaussian band fitting, enhanced printing capabilities and support for Kodak's newly released digital DC120 camera. The software allows users to annotate their images for documentation and presentation purposes. This object-oriented



Kodak's 1D image analysis software version 2.0.

layer supports a variety of custom lines, arrows and assorted shapes of any colour. Additionally, users have the ability to crop their image or create custom views of the images. A Gaussian band-finding algorithm has been incorporated into the software, which is specifically designed to improve analysis data for saturated bands, unresolved bands, or for the analysis of images with uneven backgrounds. Version 2.0 offers flexibility in printing, allowing data to be output in any one of over 16 different formats. With the 'summary' option, a user can print a single-page summary that includes the gel image, all lane information, and the analysis results for one variable of interest. In version 2.0, integrated support for Kodak's new DC120 megapixel camera is available. This new high-resolution camera offers high-quality images for the direct capture of ethidium bromide and protein gels.

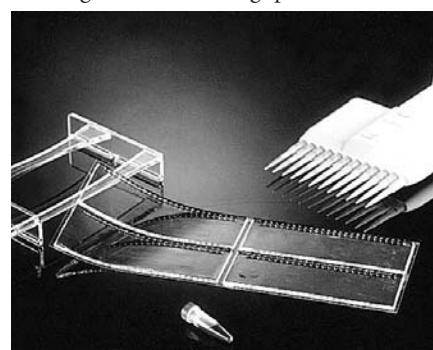
Reader Enquiry No. 107

Wide Mini Poly (NAT) gels

From Guest Elchrom Scientific

High-resolution, high-throughput precast submarine gels

These gels are intended for the analysis of DNA fragments in those applications that require high resolution and high throughput. The gels are produced in two formats and in three concentrations to cover the size range from 10 to 20,000 bp. Size differences of 1–2 per cent can be detected in the range between 60 and 2,000 bp on Wide Mini S-50 gels, which have a running path of 8.7 cm and contain 50 sample wells. For S-100 gels, the running path is 4.0 cm.



Guest Elchrom Scientific's wide precast gels.

Moreover, both S-100 and S-50 gels consist of separate 25-well sections, which can be run independently after cutting off the plastic backing to which the gel is covalently linked. Sample loading is fast with a 12-channel pipettor. The gels can be run in most existing submarine units but the improved results are often obtained when running them in Elchrom's SEA 2000, which allows the analysis of more than 500 samples per day and is said to lower cost substantially over some precast mini gels. These gels are well suited for analysing amplified DNA fragments, such as RAPD, STR and heteroduplexes.

Reader Enquiry No. 108

Using radioactivity

RayMax

From ICN

A new product line that should complement the company's radiochemical and non-isotopic labelling reagents

ICN has three types of the clear-based RayMax autoradiography film that should enable researchers to choose a film to suit the application. RayMax Universal is a double-coated, multipurpose film for general autoradiography and fluorography, offering high-resolution results at economical prices. The film consists of a clear plastic base, coated on both sides with a photographic emulsion. The emulsion is coated with a protective anti-scratch layer. This film is recommended for a multi-disciplinary laboratory where it will give high performance in a variety of applications. RayMax Beta is a single-coated, fine-grain emulsion film that gives high resolution in direct autoradiography of ¹²⁵I, ³³P, ³⁵S or ¹⁴C. It has a high silver content that is said to increase the absorption efficiency, resulting in high-speed exposures. RayMax Tritium is a single-coated film without an anti-scratch layer, which allows for direct autoradiography of tritium or ¹²⁵I. This film offers the high resolution for the detection of very weak β particles of ³H or low-energy Auger electrons associated with ¹²⁵I decay. Each type of film is available in a range of sizes to serve various applications.

Reader Enquiry No. 109

β and γ radiation products

From Ultra Violet Products

A range that includes cabinets, shields, boxes and bins

All of the products in this line are manufactured from material that effectively blocks out the most often used β isotopes of ³²P, ³⁵S, ³H and ¹⁴C, and the γ-isotopes of ¹²⁵I. The beta and gamma cabinet has been designed as a fully enclosed workstation with large access doors and a good sized working space. It can be placed on a range of laboratory trays, and when the doors are



UVP offers products for working with beta and gamma radiation.

closed, allows radioactive materials to be safely isolated. There is also a large selection of shields available in various styles and sizes, depending on the requirements of the user. The company can also provide boxes for the storage of various sizes of sample containers. With interchangeable inserts, these boxes are suited for storing Eppendorf tubes, centrifuge tubes, Falcon tubes, scintillation vials and cryotubes. There are also nine different size bins for radioactive waste.

Reader Enquiry No. 110

DNA detection and analysis

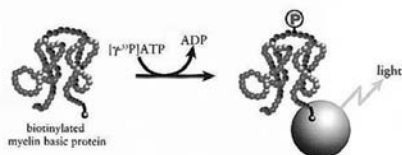
MAP kinase SPA

From Amersham Life Science

A new SPA assay for MAP kinase activity that is designed to save time in direct quantification

It is now possible to quantify directly mitogen-activated protein kinase (MAPK) activity in a high-throughput format. This follows the company's launch of MAP kinase SPA ^{33}P . Not only does the new assay offer an insight into understanding important signal transduction pathways, it is also said to be less time-consuming than conventional assays (which usually involve the capture, separation and washing of radiolabelled, phosphorylated peptides). MAPK is an important component of many signal transduction pathways, activating a multitude of intracellular molecules. The single-tube homogeneous format enables the assay to be performed in 96-well microplates and is therefore amenable to automation and high-throughput screening applications. Developed using the enzyme ERK-1, the new assay also has the potential to be used with other MAP kinases, in particular, p38

Concept for the MAP Kinase SPA assay



Amersham MAPK quantification assay.

and JNK. The assay relies on the MAPK-catalysed transfer of the γ -phosphate group of γ - ^{33}P -ATP to a 'common' substrate (biotinylated myelin basic protein, bMBP). The radiolabelled, biotinylated product is then captured onto streptavidin-coated SPA beads, bringing the label close enough to the scintillant to generate a light signal. Sufficient reagents are included to perform 500 assays.

Reader Enquiry No. 111

Transcend

From Promega

A chemiluminescent translation detection system that allows the non-radioactive detection of proteins translated in vivo

Promega states that this system offers a safer, more convenient alternative to isotopes and suffers no loss in sensitivity. The key element in these systems is the tRNA, a biotinylated lysyl tRNA. When added to an *in vitro* translation reaction the biotinylated lysine is incorporated into the newly translated protein. The protein can then be detected with streptavidin-horseradish peroxidase and Transcend chemiluminescent substrates. The chemiluminescent detection reagents are based on luminol and are said to be well suited for the detection of proteins on nitrocellulose or PVDF membranes. As lysine is more prevalent than the commonly used radiolabelled amino acid methionine, this system should prove useful for those studying proteins with few methionines or truncated proteins, as in the protein truncation test (PTT). Biotinylating the protein not only allows for its non-radioactive detection, but also for its capture either on streptavidin resins or membranes. A colorimetric detection alternative is also available.

Reader Enquiry No. 112

DU Series 500 spectrophotometers

From Beckman Instruments

Ultraviolet/visible spectrometers are designed to offer affordability for routine applications

These new spectrophotometers are said to combine easy sample handling with accurate measurement and interchangeable sampling modules that allow the user to reconfigure the system quickly and easily. Each system comes with a complete set of application programs. There are two models available: the DU 520, which delivers accurate measurements for applications such as single and multiple wavelength analysis, wavelength scanning, spectral manipulation, time-based kinetic/rate determination and single-component analysis for concentration determination, and the DU 530, which offers all these features plus application programs for protein and nucleic acid analysis, including DNA/RNA/oligonucleo-



Beckman's DU series 500 spectrometers.

tide quantification modes for determining molecular weight, concentrations and theoretical melting point.

Reader Enquiry No. 113

DNAsis

From Hitachi Software

Version 2.5 of this software is offered, as an upgrade and demonstration CDs

This newly upgraded gene analysis software program includes a direct interface to the NCBI BLAST database. Searches can be initiated within DNAsis and results opened in either a text file or as an HTML file. Primer design functions have also been added, including dimer, self-dimer, hairpin, repeat and false-priming site analysis. This version also includes a file converter, so that data may be shared between laboratories that have different DNA sequence analysis packages. All new upgrade features can be saved and printed, or imported to a favourite word processing or similar package for further manipulation. A free demonstration CD is available for this software package, which includes ScanDNAsis, an electrophoresis gel image analysis software for Macintosh computers; DNAsis for Windows and MacDNAsis provide full range sequence analysis software packages; and HybSimulator allows primer design and simulation for Macintosh or Windows platforms.

Reader Enquiry No. 114

BioFocus LIF²

From Bio-Rad

A dual-laser-induced fluorescence detector

The new BioFocus LIF² detector for capillary electrophoresis has dual detection and improved sensitivity. Because the detector allows excitation with two separate, high-intensity lasers and simultaneous dual detection of emitted fluorescence, it provides much greater flexibility and allows higher productivity, as the unknown sample and the standard can be run at the same time for optimal internal referencing. Stated to be 1,000 times more sensitive than conventional ultraviolet absorbance detectors, this detector can detect picomo-



The BioFocus LIF² dual-laser-induced fluorescence detector from Bio-Rad.

lar concentrations of DNA. The system is available in both single- and dual-laser configurations, and is compatible with a range of external lasers to meet specific application requirements.

Reader Enquiry No. 115

Anti-Lewis^x monoclonal antibody

From Dako

A monoclonal antibody that should assist in apoptosis detection

Le^x is an oligosaccharide connected with other Lewis substances: Le^a, Le^b and Le^x. Although its function is not fully understood, Le^x has been identified phenotypically as a marker of specific cell types and possibly of specific stages of cell differentiation. Recently, Le^x was found to be a marker characteristically present on cells undergoing apoptosis. There is said to be a good correlation between Le^x expression and DNA fragmentation, as measured by 3'-OH-end labelling. There is no expression of Le^x on cells undergoing differentiation or necrosis. The new monoclonal antibody is derived from clone BM1, which is said to work well in formalin-fixed, paraffin-embedded tissue sections, and should be useful in studies of the apoptotic process. A large number of other antibodies connected with apoptosis, including

anti-bcl-2 and anti-p53, are also available from Dako.

Reader Enquiry No. 116

XL10-Gold ultracompetent cells

From Stratagene

Plasmid libraries that are now said to approach the quality of lambda libraries

Stratagene states that these new cells were derived to provide the highest transformation efficiencies available for ligated DNA, stated to produce up to 25-fold more primary clones than DH10B cells. Plasmid libraries constructed in XL10-Gold cells may prove to be larger and more complex, approaching the quality of lambda libraries. Stratagene has developed three plasmid/cDNA synthesis kits: the pBlue-script II XR library construction kit for prokaryotic cloning, the pCMV-Script XR library construction kit for high-level eukaryotic expression and the pAD-GAL42.1 XR library construction kit for two-hybrid screening of yeast. All three kits are provided with sufficient reagents for five cDNA synthesis reactions, a predigested vector and XL10-Gold ultracompetent cells. Each kit is quality controlled to produce 1×10^6 primary clones with test mRNA.

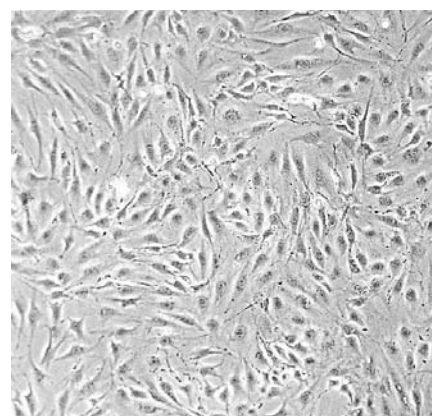
Reader Enquiry No. 117

TransIT

From PanVera

Reagents for efficient DNA and RNA transfections

These reagents are said to offer economical, high-efficiency transfection with minimal cellular toxicity in both stable and transient transfections. The products also offer reproducibility, long shelf life and straightforward optimization. This combination of features makes TransIT reagents well suited for all gene expression studies where the post-transfection state of the cell is important. TransIT reagents



PanVera's TransIT reagents for high-throughput screening or high volume protein production.

can also be used for high-throughput transfection screening or high-volume protein production.

Reader Enquiry No. 118

RetroXpress

From Clontech

A new retroviral gene expression system

RetroXpress has been formulated to accomplish efficient retrovirus-mediated gene transfer and expression. The kit includes the RetroPack PT67 packaging cell line and RetroXpress vectors for introducing genes into a wide variety of mammalian cell types (*in vitro* or *in vivo*). The PT67 packaging cell line contains stably integrated retroviral structural genes for the safe production of high titres of infectious, replication-incompetent retroviruses. Transduction of the recombinant retroviral particles is said to result in nearly 100 per cent of cells expressing the gene of interest.

Reader Enquiry No. 119

These notes are compiled by Brendan Horton from information provided by the manufacturers. For more details, fill in the reader service card bound inside the journal.

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